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THE NATURE OF REACTION WOOD

II. THE CELL WALL ORGANIZATION OF COMPRESSION WOOD TRACHEIDS

By A. B. WARDROP* and H. E. DADSWELL*

[Manuscript received November 14, 1949]

Summary

Optical and X-ray methods have been used in the examination of the secondary cell wall of compression wood tracheids from a number of species of gymnosperms. By these methods it has been shown that the cell wall of compression wood tracheids consists of two layers. In the outer layer the micelles are inclined at a large angle to the longitudinal axis of the tracheid, while in the inner layer the micelles are inclined at a relatively smaller angle. In the inner of the two layers there exist radial discontinuities in the spiral micellar structure, which are visible as longitudinal striations in the cell wall. These discontinuities also account for the radial distribution of lignin which is observed in transverse sections of compression wood tracheids. By determining the average tracheid length of the last-formed late wood in the various growth rings of several eccentric stems of *Pinus radiata* D. Don it has been shown that the tracheids of compression wood are appreciably shorter than would be the case if no compression wood were present. A study of the change in micellar orientation with change in tracheid length has indicated that the angle of micellar orientation in compression wood tracheids does not differ significantly from that existing in normal wood tracheids of similar length. In so far as the properties of wood are determined by cell wall organization, it is concluded that comparisons between compression wood and normal wood should be made on material of the same tracheid length and spiral organization. It is suggested that both the reduction in tracheid length and eccentric radial growth in stems containing compression wood are to be attributed to an increase in the number of both transverse and tangential longitudinal divisions of the fusiform initials of the cambium.

I. INTRODUCTION

A study of the cell wall organization of the tracheids from the compression wood of gymnosperms is of interest, both from the way in which the organization of the normal cell wall has been modified in response to a gravitational stimulus, and from the way in which the properties of the wood change as a result of this modification. Furthermore, the greater anatomical simplicity of the gymnosperms provides a more favourable material for the study of these changes than the corresponding reaction wood (tension wood) of angiosperms, discussed in the first paper of the series (Wardrop and Dadswell 1948). The macroscopic appearance of compression wood and the anatomical details of its constituent tracheids (rounded form and associated intercellular spaces, cell walls somewhat thicker than normal with marked longitudinal spiral striations, and shorter length) have been referred to by numerous workers (Pillow and Luxford 1937;

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Münch 1937-38; Jaccard 1938). The influence of compression wood on the properties of timber has also been described (Pillow and Luxford 1937; Desch 1947; Brown, Panshin, and Forsaith 1949; Dadswell and Wardrop 1949). Chemically, compression wood is relatively higher in lignin content and lower in cellulose than normal wood (Johnson and Hovey 1918; Dadswell and Hawley 1929) and it has been shown by Bailey and Kerr (1937) and Dadswell and Ellis (1940) that the lignin distribution in the cell walls of compression wood tracheids is in a characteristic radial pattern when viewed in cross section in contrast to the more normal radio-concentric pattern of normal wood tracheids.

The secondary wall of the normal tracheids of gymnosperms consists of three coaxial micellar spirals (see Fig. 1(A)) recognizable, when cross sections are examined microscopically between crossed Nicols, as three layers of different optical properties. The micellar organization of these layers is such that, with reference to the longitudinal tracheid axis, the central layer (S2) consists of a steep micellar spiral while the outer layer (S1) and the inner layer (S3), which is adjacent to the lumen, possess a relatively flat micellar spiral (Kerr and Bailey 1934; Wardrop and Preston 1947). The inner layer (S3), however, is not always present. If this conception of the cell wall organization of normal tracheids is accepted, it is pertinent to know in what ways compression wood tracheids differ from the typical organization.

An examination of the literature suggested that the following aspects of the organization of compression wood tracheids required further investigation:

- (i) whether all three of the layers of the secondary wall, S1, S2, and S3, occur in compression wood tracheids (Münch reports only two),
- (ii) the significance of the radial lignin pattern visible in cross section,
- (iii) the nature of the characteristic spiral striations of the cell wall visible in longitudinal section, and
- (iv) the significance of the observation that compression wood tracheids are shorter than comparable normal tracheids.

Each of these aspects will be discussed in the present paper, but it is proposed to refer particularly to the way in which the cell wall organization of compression wood tracheids is related to their length.

It has been well known since the time of Sanio (1872) that the average tracheid length in stems of gymnosperms increases rapidly over successive annual rings during the first 20-50 years of growth and thereafter ceases to increase or increases only slowly. The fundamental investigations of Preston (1934, 1948) have indicated that the micellar spiral organization of the central layer (S2) of the secondary wall can be related to the tracheid length by an equation of the form:

$$\cot \theta = a + bL, \quad \dots \dots \dots (1)$$

where L is the tracheid length, θ is the angle between the tangent to the direction of the micelles and the longitudinal axis of the tracheid, and a and b are constants. A similar equation has also been applied to the outer layer (S1) of the secondary wall (Preston and Wardrop 1949).

From these investigations it is obvious that, as the tracheids increase in length over successive growth rings, the micellar spiral angle θ becomes smaller, and that short tracheids will possess a relatively flatter micellar spiral angle than long tracheids. This is of considerable significance in relation to compression wood tracheids since it has been reported (Pillow and Luxford 1937; Münch 1937-38) that these tracheids are shorter than comparable normal wood tracheids and that they possess a flat spiral micellar organization. Thus the question is raised as to whether the spiral micellar organization of compression wood tracheids is related to tracheid length in the same manner as is represented by equation (1) or in some other way. Moreover it was felt that some confirmatory evidence regarding the length of compression wood tracheids in relation to normal tracheids was desirable, as statements on this point in the literature are not supported by data, nor do they indicate how comparisons were made. This is important in view of the known variations in tracheid length with distance from pith, and with height in tree. It was therefore decided to examine the changes in tracheid length and associated changes in spiral micellar orientation in eccentric stems containing both normal and compression wood. The results of these investigations together with additional information on points (i) to (iv) above are given in the following sections of this paper.

II. EXPERIMENTAL

(a) Details of Cell Wall Organization

Observations were made on compression wood from a variety of endemic and imported species of gymnosperms, including the following:

<i>Pinus radiata</i> D. Don.	} from plantations
<i>Pinus pinaster</i> Soland.	
<i>Pseudotsuga taxifolia</i> (Lamb.) Britt.	} branch material from trees growing in the Melbourne Botanical Gardens
<i>Taxus baccata</i> L.	
<i>Taxodium distichum</i> (L.) Rich.	
<i>Araucaria cunninghamii</i> Ait.	
<i>Araucaria bidwilli</i> Hook.	
<i>Athrotaxis selaginoides</i> D. Don.	
<i>Callitris tasmanica</i> Baker and Smith.	
<i>Dacrydium franklinii</i> Hook. f.	
<i>Diselma archeri</i> Hook. f.	
<i>Phaerosphaera hookeriana</i> Archer.	
<i>Phyllocladus asplenifolius</i> (Labill.) Hook.	

Examination of thin cross sections by means of the polarizing microscope using crossed Nicols revealed two definite layers of the secondary wall, both of which showed considerable birefringence. These two layers appeared to be comparable with layers S1 and S2 of Figure 1(A), the layer comparable with S3 being usually absent or only poorly developed (Plate 1, Fig. 1). The birefringence of layer S2 was rather less than that of the outer layer, indicating that, in

this layer, there was a relatively steeper spiral micellar organization than in the outer layer. The sign of birefringence of both of the layers was positive with respect to the cell surface, indicating that the micelles were parallel to the surface.

Visual evidence of the micellar orientation in the two layers has been obtained by crushing isolated fragments of compression wood tracheids from hoop pine (*Araucaria cunninghamii*) in the manner described in the first paper of this series (Wardrop and Dadswell 1948). Part of such a tracheid is shown in Plate 1, Figure 3, in which two distinct directions of striations can be seen—one being almost transverse to the longitudinal tracheid axis, and the other inclined at approximately 45° to this axis. The dichroic properties of these striations made it apparent that they corresponded to true directions of micellar orientation. The characteristic striations normally visible without crushing were very coarse and easy to distinguish in the various species of the genus *Pinus* (cf. Jaccard 1940), but in certain other specimens examined, e.g. *Diselma archeri*, *Phaerosphaera hookeriana*, *Araucaria cunninghamii*, they were extremely fine, although visible under high power. Examination of these striations indicated that there was apparently no other cell wall material between them, since they were often displaced in sectioning (Plate 1, Fig. 4). This observation was confirmed by cutting sections at angles approximately parallel to the direction of the striations when it could be seen that they were ribs of cell wall substance extending through the layer S2 of the secondary wall (Plate 1, Fig. 5).

X-ray examination of various specimens of compression wood was carried out with the beam perpendicular to the tangential face and using $\text{CuK}\alpha$ radiation and a specimen film distance of 3 cm. (cf. Bailey and Berkley 1942). The spread of the equatorial arcs yielded a value for the angle of the micellar orientation in agreement with that obtained by optical observations (Plate 1, Fig. 6).

In referring to the layers of the secondary wall of compression wood tracheids, Bailey and Berkley have reported that, in *Taxodium distichum* (L.) Rich., there is an intervening layer which is isotropic or feebly birefringent between the two wide layers normally present. They stated that the optical properties of this layer are due—"not to a longitudinal orientation of fibrils, but to the fact that this layer is composed largely of non-cellulosic constituents." In the present investigation, no obvious isotropic layer similar to that depicted by Bailey and Berkley was observed in *Taxodium distichum*. However, in *Pinus pinaster*, *Pinus radiata*, and *Pseudotsuga taxifolia* there was some evidence of an isotropic layer between the two main layers of the secondary wall of compression wood tracheids (Plate 1, Fig. 2), but it was not possible to decide whether this was of the type referred to by Bailey and Berkley.

(b) Lignin Distribution in the Cell Wall

In transverse section the distribution of lignin as demonstrated by means of treatment with 72 per cent. sulphuric acid (Dadswell 1931) was observed to be in a radial pattern. This is in contrast with the radio-concentric lignin pattern for normal wood tracheids. Those specimens with coarse spiral striations in the

cell wall, e.g. *Pinus pinaster*, showed a coarse lignin pattern in cross section (Plate 2, Fig. 1); on the other hand, those with fine spiral striations showed a fine lignin pattern in cross section (Plate 2, Fig. 3). Longitudinal sections treated in the same way gave a similar correlation between striations and lignin pattern (Plate 2, Fig. 2). In a specimen of *Araucaria cunninghamii* two striation directions in the lignin pattern were observed when the focus was altered slightly; these presumably correspond to the two layers of the cell wall referred to above and are illustrated in two adjacent tracheids in Plate 2, Figure 4.

(c) *Variation of Tracheid Length and Micellar Organization of the Cell Wall in Stems containing Compression Wood*

For this part of the investigation cross sections of logs taken from three specimens of *Pinus radiata* approximately 20 years old were obtained. Each of these showed compression wood on one side. One was used for the determination of the tracheid length, at 60° intervals around the stem in the late wood of the ninth growth ring, which contained compression wood. The average tracheid length in each case was determined by the measurement of 50 tracheids isolated from longitudinal sections cut from the last-formed late wood. The

TABLE 1

VARIATION IN TRACHEID LENGTH AT 60° INTERVALS AROUND THE NINTH GROWTH RING OF A SPECIMEN OF *PINUS RADIATA* D.DON

Radius	Tracheid Length (mm.)	Ring Width (cm.)	Comments
1 (0°)	3.58	0.80	Comp. wood absent
2 (60°)	3.15	0.80	Comp. wood absent
3 (120°)	2.94	0.95	Comp. wood absent
4 (180°)	2.74	1.20	Comp. wood present
5 (240°)	2.79	1.20	Comp. wood present
6 (300°)	3.39	0.85	Comp. wood absent

results, set out in Table 1, indicate the variation in tracheid length obtained. Ring width and presence or absence of compression wood are also recorded. In the other two specimens a strip of wood was taken along a diameter, in such a manner as to pass through the compression wood bands occurring on one side of the cross sections. The average angle of micellar orientation was determined in the last-formed late wood of the different growth rings from the degree of spread of the 002 diffraction arcs in the X-ray diagrams. The values so obtained corresponded to that existing in layer S2 of the cell wall (Plate 1, Fig. 6). After X-ray examination the specimens of late wood from each ring were macerated and the average tracheid lengths determined. Results of these determinations, together with the corresponding angles of micellar orientation, information on presence or absence of compression wood, and ring width, for both sides of the stem, are shown in Table 2.

TABLE 2
 VARIATION IN TRACHEID LENGTH, MICELLAR SPIRAL ANGLE, AND RING WIDTH IN
PINUS RADIATA D.DON

Specimen	Age in Years	Wide Side			Narrow Side		
		Tracheid Length (mm.)†	Micellar Spiral Angle (degrees)	Ring Width (cm.)	Tracheid Length (mm.)†	Micellar Spiral Angle (degrees)	Ring Width (cm.)
1	1	1.28	53	0.74	—	48	0.60
	2	1.56	—	0.66	1.64	—	0.80
	3	1.77	48	1.00	2.07	50	1.00
	4	2.10	—	0.80	2.13	—	0.60
	5	2.36	45	0.60	2.73	—	0.50
	6	2.84	—	0.80	2.90	39	0.60
	7	2.84	41	0.80	2.72	—	0.40
	8	3.27	—	0.50	3.07	35	0.40
	9	3.13	36	0.70	3.11	26	0.40
	10°	2.61	40	0.80	3.35	29	0.40
	11°	2.53	35	0.60	3.39	31	0.40
	12°	2.89	42	0.70	3.37	29	0.30
	13°	2.92	37	0.80	3.18	24	0.50
	14	3.18	27	0.60	3.30	23	0.10
	15	3.03	—	0.80	3.43	—	0.30
	16	3.35	35	0.70	3.39	25	0.30
	17	2.88	—	1.00	3.52	—	0.30
	18	3.19	—	1.10	3.48	—	0.30
	19	2.96	25	0.80	3.57	23	0.20
	20	3.05	—	0.70	3.50	—	0.30
2	1	1.72	34	1.60	—	—	1.50
	2	—	—	1.35	2.61	31	1.60
	3	2.56	32	1.80	2.71	35	1.85
	4	—	—	1.50	—	—	1.40
	5	3.14	30	1.35	3.33	23	1.25
	6	3.54	24	1.20	—	—	0.85
	7	3.70	24	1.20	3.72	14	0.65
	8	3.89	22.5	1.00	—	—	0.25
	9°	3.98	27	0.85	4.02	13.5	1.00
	10°	2.47	32	1.00	—	—	0.75
	11°	2.67	37	1.05	4.28	15	0.65
	12°	3.13	35	1.05	—	—	0.60
	13°	2.70	22.5	1.00	4.23	16	0.45
	14°	—	—	1.00	—	—	0.60
	15°	3.15	31	0.85	4.40	16	0.50
	16°	—	—	0.95	—	—	0.40
	17°	2.97	33.5	0.75	4.42	12	0.50
	18°	—	—	1.00	—	—	0.55
	19	3.59	28	1.10	4.51	13	0.50

† Average of 50 measurements.

° Compression wood present.

III. DISCUSSION

From a consideration of the above evidence it will be apparent that the secondary wall of compression wood tracheids usually consists of only two layers which, from the disposition of the micellar spirals, might be regarded as analogous to the layers S1 and S2 of normal tracheids (Fig. 1(A)). The inner layer corresponding to S3 of the normal tracheids is absent or only feebly developed. The existence of an isotropic non-cellulosic layer between the layers S1 and S2 reported by Bailey and Berkley (1942) cannot be regarded as a characteristic of these tracheids. However, the inner of the two layers normally present in compression wood tracheids differs from the layer S2 of the normal tracheids. There exists a radial discontinuity of structure such that this layer can be imagined to consist of spirally arranged ribbons of cell wall material, the longer, narrow edge of which is attached to the adjacent cell wall layer. From the X-ray and optical evidence it is apparent that the micelles lie parallel to the length of these spiral bands. In terms of this structure it can be seen that the individual cells would appear to have a radial lignin distribution when examined in cross section (Plate 2, Figs. 1 and 3) and a spiral distribution in longitudinal section (Plate 2, Figs. 2 and 4).

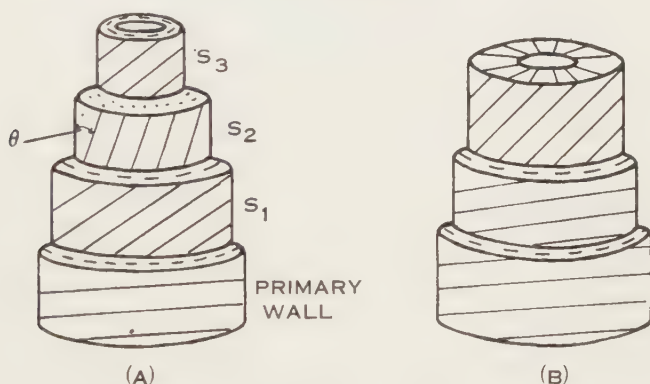


Fig. 1.—Diagrammatic representation of the cell wall organization in (A) a normal tracheid and (B) a compression wood tracheid. The lines represent the directions of spiral micellar orientation only. The texture of the primary wall ("tube structure") differs from that of the secondary wall layers ("fibrous structure").

On the basis of such a model it is also apparent that the optic sign of the wall would be positive with respect to the cell surface. Thus, in contrast to a normal wood tracheid (Fig. 1 (A)), the cell wall organization of a compression wood tracheid can be represented by Figure 1 (B). As with normal wood tracheids, no evidence of the layer S1 was observed in the X-ray examination of compression wood tracheids. It has been pointed out by Preston (1946) that the meridional arcs (Plate 2, Fig. 6) are to be interpreted as arising from the fusion of the 002 equatorial arcs in specimens of relatively large micellar spiral angle. The fact that the contribution of the outer layer is not apparent in the

diffraction diagram is most probably due to the existence of considerable micellar angular dispersion and to diffuse scattering from other cell wall substances present in this layer (Wardrop and Preston 1947). A further factor is the decrease in the angle of the micellar spiral organization, which takes place in both of these layers with increasing cell length and makes difficult the detection of any contribution from the outer layer (Preston and Wardrop 1949).

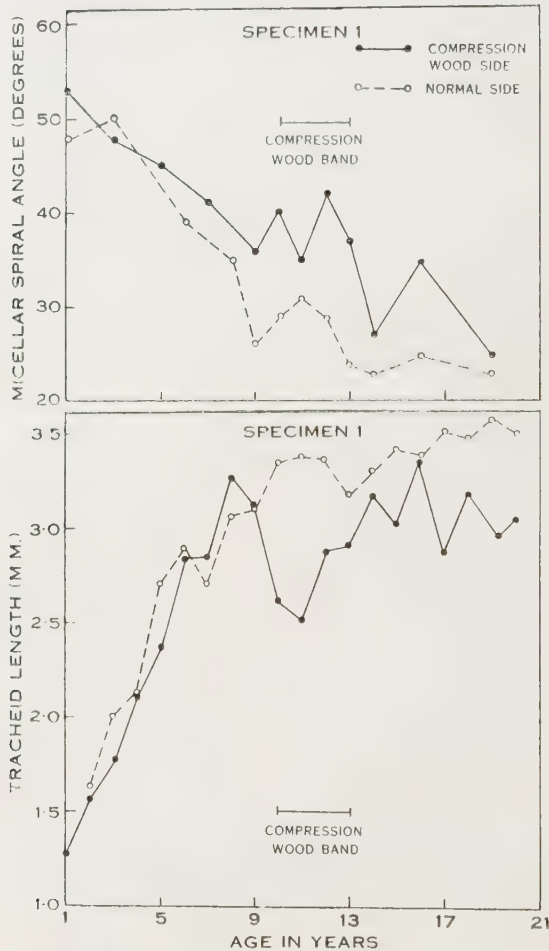


Fig. 2.—Variation of tracheid length and micellar spiral angle with age in *Pinus radiata* D. Don.

It can be seen from Table 2 and Figure 2 that the formation of compression wood is accompanied by a marked decrease in tracheid length and increase in spiral angle. After this sudden change, the tracheid length again increases and the spiral angle again decreases. On the side of the stem opposite to the compression wood, and prior to the formation of compression wood on the side containing it, the tracheid length increases from the pith outwards in the usual

manner (Sanio 1872 and others). Where no compression wood is present this increase is maintained to the eleventh ring (Fig. 2), after which the length attains a more or less constant value. Correspondingly, the spiral angle gradually decreases from the pith outwards, and again where no compression wood is present, it attains a more or less constant value at approximately the thirteenth ring in one specimen and the sixteenth ring in the other. Thus it is evident that the tracheids in the compression wood area are appreciably shorter than they would have been if no compression wood were present, and that this shorter length is accompanied by an increase in spiral angle.

In order to determine whether the micellar organization in compression wood tracheids is significantly different from that in normal wood tracheids, use was made of the relationship developed originally by Preston (1934) and referred to in equation (1) above. The tracheid length and the corresponding value for the cotangent of the spiral angle were plotted for both normal and compression wood growth rings (see Fig. 3). Straight lines were fitted by the method of least squares, firstly to the normal and compression wood results separately, and then to the combined data. Statistical examination showed that the single line gave a satisfactory fit, indicating that the assumption of a common relationship between tracheid length and the cotangent of the spiral angle for both normal wood and compression wood was in accordance with the data.

It was therefore concluded that, so far as the angle of micellar orientation of the layer S2 of the cell wall is concerned, compression wood tracheids are apparently no different from normal wood tracheids of the same length. This conclusion may be of considerable practical value, as compression wood properties are usually compared with properties of adjacent normal wood. If, as seems probable, properties of timber are determined by the cell wall organization (e.g. shrinkage, swelling, strength-properties) then it is clear that comparison should be made between compression wood and wood of similar tracheid length and micellar angle. Thus, from this point of view, compression wood may be regarded as physiologically younger than adjacent normal wood. In this connection it is of interest to note that some preliminary work on *Pseudotsuga taxifolia* (Wardrop 1948) has shown that the Cross and Bevan cellulose content increases in successive rings from the pith; thus, in this specimen a low cellulose content was associated with a shorter tracheid length and relatively large spiral angle. It is therefore significant that compression wood with its short tracheids and associated large spiral angle has also been found to contain less cellulose than corresponding normal wood.

Again, if as has been suggested for wood and demonstrated for cotton (Meredith 1946), the micellar organization is a factor contributing to properties such as tensile strength and possibly shrinkage and swelling, then compression wood might be expected to more nearly resemble in properties the wood near the centre of the stem than that immediately adjacent to it. Support for this view is seen in the facts that compression wood is known to be characterized by high longitudinal shrinkage and that the recent investigations of Pillow (1949), as well as observations in this laboratory, have demonstrated the existence of

high longitudinal shrinkage near the centre of the stem. The poor tensile strength of compression wood in comparison with normal wood adjacent to it may also be explained in the above terms, and experiments along these lines are already in progress in this laboratory.

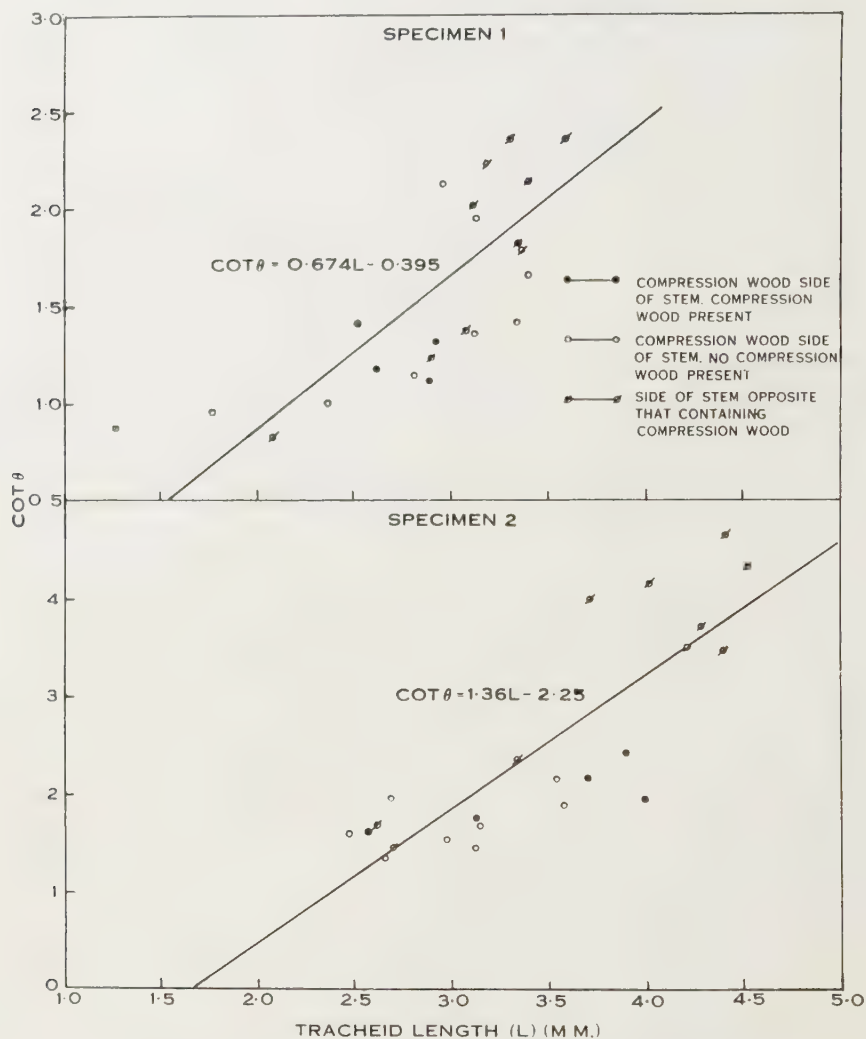


Fig. 3.—Relation between angle of micellar orientation (θ) and tracheid length (L) in *Pinus radiata* D. Don.

It is well known that compression wood formation is usually associated with eccentric growth in the stem (Jaccard 1938), and, as can be seen from Figure 2, considerable reduction in tracheid length also takes place. These points are of interest in relation to some considerations put forward by Priestley (1930), who pointed out that vigorous radial growth would mean rapid tangential divisions of the fusiform initials of the cambium. If this were to result in a postponement

of transverse divisions in the cambium, then a wide annual ring would be associated with the formation of long tracheids. If, however, rapid tangential divisions are accompanied by an increased number of transverse divisions, then a wider annual ring would be correlated with the formation of shorter tracheids. This latter possibility obviously seems to present a reasonable explanation of the eccentric growth and shorter tracheid length which accompanies compression wood formation. Support for this view can be seen in Table 1 where the tracheid length and ring width at 60° intervals around one growth ring containing compression wood are listed.

The factors leading to the increased rate of cell division resulting in compression wood formation in stems and branches under gravitational stimulus are not known. The recent experiments of Wershing and Bailey (1942), in which compression wood formation was induced in conifer seedlings with 3-indoleacetic acid, would, however, strongly suggest the operation of some correlative factor such as a redistribution of auxin in the stem, resultant upon the operation of the gravitational stimulus.

In conclusion, it will be apparent that the cell wall organization in compression wood tracheids is to be regarded as a modification of the structure present in normal tracheids, and that this structure is determined by the shorter length of the cells. Although it is probable that this shorter cell length is governed by an increase in the number of transverse divisions occurring in the fusiform initials of the cambium, the way in which the course of cell division in the cambium is governed by the gravitational stimulus remains to be demonstrated.

IV. ACKNOWLEDGMENTS

The authors wish to acknowledge their indebtedness to Dr. R. D. Preston, Reader in Plant Biophysics, Leeds University, for permission to use X-ray equipment in his laboratory, for some of the X-ray determinations of micellar orientation, and for helpful discussions of the problem; to Mr. I. J. W. Bisset for organizing the measurement of tracheid length for specimen 1; to the Director of the Melbourne Botanical Gardens for certain of the specimens examined; to Mr. F. Schenk for donating specimen 1; and to Mr. E. J. Williams of the Section of Mathematical Statistics, C.S.I.R.O., for assistance in the mathematical analysis of the results.

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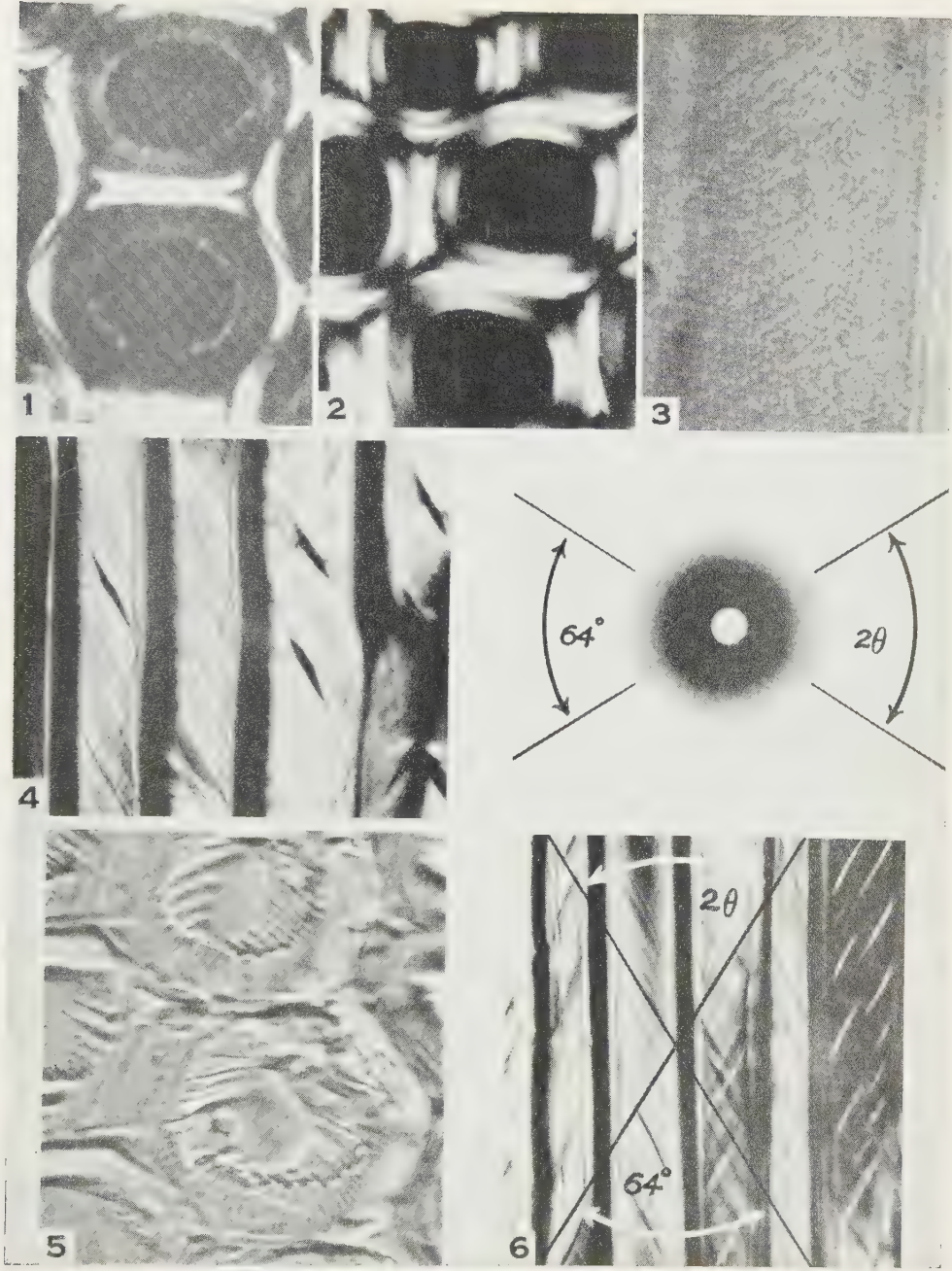
EXPLANATION OF PLATES 1-2

PLATE 1

- Fig. 1.—*Pinus pinaster*. Transverse section of compression wood photographed between crossed Nicols showing feeble development of the inner layer of the secondary wall. $\times 930$.
 Fig. 2.—*Pseudotsuga taxifolia*. Transverse section of compression wood photographed between crossed Nicols showing the isotropic layer described by Bailey and Berkley (1942) (see text). $\times 930$.
 Fig. 3.—*Araucaria cunninghamii*. Part of the delignified cell wall of a compression wood tracheid after staining with congo red and crushing. Note the two striation directions. $\times 930$.
 Fig. 4.—*Pinus pinaster*. Radial longitudinal section ($15\ \mu$) of compression wood, photographed between crossed Nicols showing distortion of the pits and spiral striations. Note the displacement of the part of the cell wall in lower part of the centre tracheid. $\times 410$.
 Fig. 5.—*Pinus pinaster*. Section of compression wood ($15\ \mu$) cut approximately parallel to the striation direction and illustrating radial discontinuities of the secondary wall. $\times 930$.
 Fig. 6.—*Pinus pinaster*. Tangential longitudinal section ($20\ \mu$) of compression wood photographed between crossed Nicols ($\times 410$), and the X-ray diffraction photograph of the same specimen. The intense blackening of the X-ray film surrounding the central spot arises from the diffuse scattering which is here exaggerated because of the prolonged exposure with such a thin specimen.

PLATE 2

- Fig. 1.—*Pinus pinaster*. Transverse section ($20\ \mu$) after treatment with 72 per cent. sulphuric acid, showing radial distribution of lignin in the cell wall. $\times 980$.
 Fig. 2.—*Pinus pinaster*. Tangential longitudinal section after treatment with 72 per cent. sulphuric acid, showing spiral arrangement of lignin in the cell wall. $\times 430$.
 Fig. 3.—*Araucaria cunninghamii*. Transverse section ($20\ \mu$) after treatment with 72 per cent. sulphuric acid, showing fine radial distribution of lignin in the cell wall. Compare Figure 1. $\times 980$.
 Fig. 4.—*Araucaria cunninghamii*. Tangential longitudinal section ($20\ \mu$) after treatment with 72 per cent. sulphuric acid, showing differences in striation direction in the lignin pattern of two adjacent cells. Compare Figure 2. $\times 980$.



WARDROP AND DADSWELL.—THE NATURE OF REACTION WOOD. II



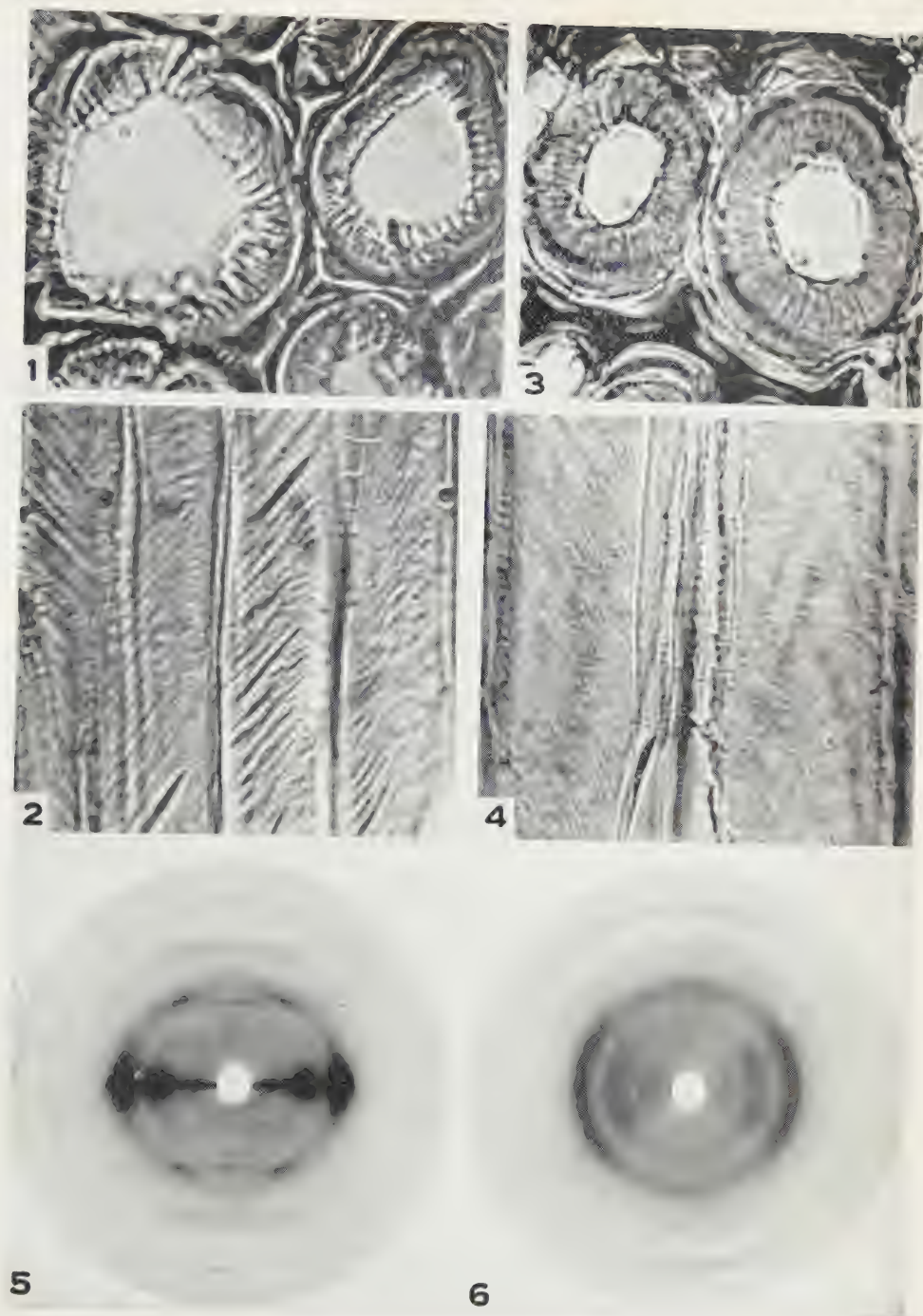




Fig. 5.—*Pinus radiata*. X-ray diffraction diagram of normal wood from the eleventh annual ring of Specimen 2, taken with the X-ray beam parallel to the medullary rays, using $\text{CuK}\alpha$ radiation and a specimen film distance of 3.7 cm. Note the small spread of the equatorial arcs indicating a steep spiral micellar orientation $\theta = 15^\circ$. Compare Figure 6.

Fig. 6.—*Pinus radiata*. X-ray diffraction diagram of compression wood from the eleventh annual ring of Specimen 2, taken with the X-ray beam parallel to the medullary rays using $\text{CuK}\alpha$ radiation and a specimen film distance of 3.7 cm. Note the large angular spread of the equatorial arcs indicating a flat spiral micellar orientation $\theta = 37^\circ$. Compare Figure 5.

THE DISTRIBUTION OF ZINC IN OAT PLANTS

By J. G. WOOD* and PAMELA M. SIBLY*

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Summary

Three experiments are described in which oat plants (*var.* Mulga) were grown in Laffer sand or culture solutions containing varying amounts of added zinc. In each case, zinc contents of living and dead leaves, stems, roots, inflorescence, and grain were determined at intervals until final harvest.

Zinc was absorbed continuously throughout the life cycle of the plant. In the soil used, much of the zinc, whether native or added, was unavailable, and large increases in the amount added to the soil did not result in proportional increases in amount of zinc in the plant. It is shown that there is a threshold value for grain production in oats, but large increases in zinc contents of the plants did not cause proportionate increases in yield either of dry matter or of grain. Symptoms of zinc deficiency appeared on leaves when the concentration of zinc was less than 20 parts per million of dry matter at a time prior to exertion of the inflorescence.

Leaves contained 20-30 per cent. of total zinc in the plant. This zinc was not translocated from the leaves during senescence, but remained within the dead leaves; nor could zinc be removed by dialysis of macerated leaf material. Of the total zinc in the leaves, 15 to 20 per cent. was localized in the chloroplasts, though in spinach about 50 per cent. of the total leaf zinc occurs in the chloroplasts.

The amount of zinc in the roots increased in amount up to the time of grain development, but thereafter decreased and was translocated to other organs. The greater part of the zinc in grain and inflorescence was accounted for by uptake from the medium.

I. INTRODUCTION

Throughout large areas of South Australia, cereals and pasture plants cannot absorb from the soil sufficient zinc and copper for normal growth and development. Riceman and Anderson (1941) showed that addition of zinc in the presence of copper increased the yield of grain in oats grown on the calcareous Robe sand; and Riceman (1945) that on Laffer sand the yield of oats was increased by dressings of zinc in the presence of applied phosphate.

Reed (1939) and Reed and Beck (1939) grew maize plants in a zinc-deficient Californian soil and showed that production of cobs and kernels was depressed more than that of stalks and leaves. Reed (1942) showed, for peas and beans grown in water culture, that there was a threshold value for zinc below which plants produced only small, seedless pods; above this threshold value the weight of seeds bore a close relation to the amount of zinc supplied. Tops and roots of peas grown in solution containing 0.005-0.2 part per million

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Zn did not show significant differences in dry weight, but with beans grown in the same range of concentrations small increases in dry weight occurred with increasing concentrations of zinc. With *Andropogon sorghum*, however, there was a threshold value for development of tops and heads.

Systematic determinations of amount of zinc in plant tissues have been few and restricted chiefly to contents at time of flowering or at final harvest, with the object of determining the minimum zinc contents required for normal development. Piper and Walkley (1943) concluded from the percentage distribution of zinc in grain and straw of oat plants towards the end of the life cycle that zinc was translocated from straw to grain as maturity approached.

However, analyses at time of harvest can give little information about uptake and translocation during development. As pointed out by Gregory (1937), growth in cereals is an exponential process, determined by development of new meristems, during which the external supply of a nutrient in minimum is depleted and its rate of supply to the plant falls. When demand by the plant for the nutrient overtakes supply, internal starvation occurs and towards the end of the life cycle plants tend to possess the same concentration of the nutrient on a dry weight basis; this is low and may be independent of the magnitude of the original external supply. In this paper the authors report data for dry weights and zinc contents of the different plant organs of oat plants at different stages of development of the plant, the plants themselves being under a variety of conditions. From these data, movements of zinc within the plant are traced.

II. EXPERIMENTAL

(a) *Description of Experiments*

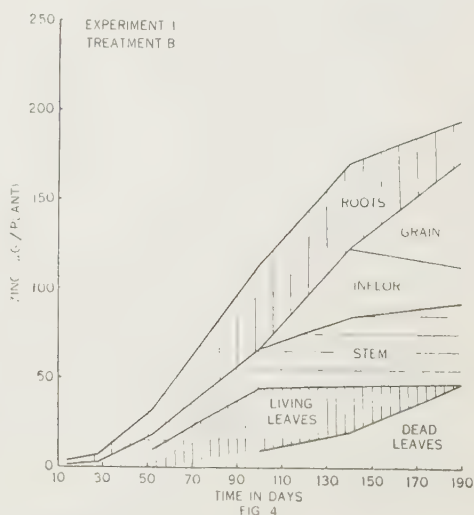
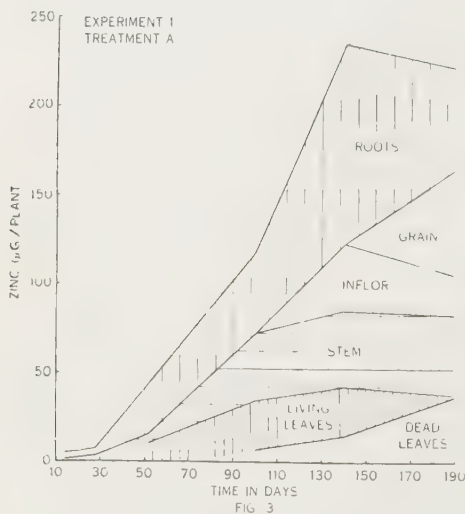
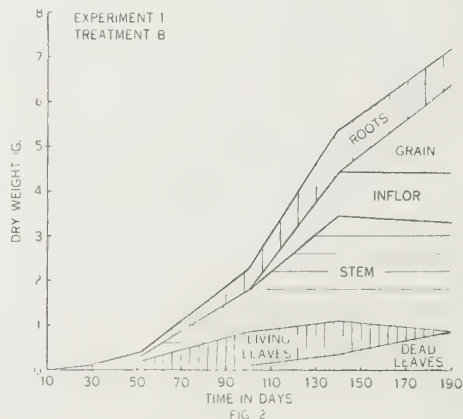
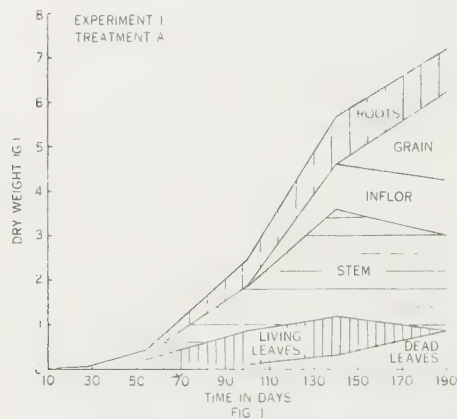
For each experiment, plants of a pure line of oats, *Avena sativa* var. Mulga, were grown in jars in a glass house, care being taken by use of paper shields that leaves did not come in contact with the soil.

Experiment 1.—Plants were grown in glazed earthenware pots, each containing 3 kg. Laffer sand obtained from an area which had been cropped once previously with a legume which showed symptoms of zinc deficiency. Six de-husked grains were sown in each pot on April 28, 1946 and thinned to three even plants per pot 9 days after sowing. Additional seedlings were grown for the first harvest but on subsequent occasions three pots from each treatment were harvested.

All pots received the following nutrients (in grams per pot) eleven days after sowing: $\text{Ca}(\text{H}_2\text{PO}_4)_2$, 0.5g.; NaNO_3 , 1.0g.; KCl , 0.4g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.14g.; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.015g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.01g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.04g.; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.003g. An additional 1.0g. NaNO_3 per pot was added 94 days after sowing.

Prior to addition to the sand, solutions of NaNO_3 , KCl , and MgSO_4 were freed from zinc by extraction with dithizone and removal of excess of the latter. $\text{Ca}(\text{H}_2\text{PO}_4)_2$ was prepared from CaCl_2 and KH_2PO_4 solutions after removal of zinc by dithizone extraction. Fe, Mn, Cu, and Mo salts were prepared by recrystallizing AR grade chemicals.

The pots were divided into two series according to the zinc treatment designed for them, viz. pots of Treatment A received 0.01g. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (2300 μg . Zn) per pot; those of Treatment B received no additional zinc. The pots were maintained at 60 per cent. water-retaining capacity of the soil by frequent additions of water redistilled from a Pyrex glass still.



Figs. 1-4.—Dry weights (g. per plant) and absolute amounts of zinc in plant organs, Experiment 1.

Appropriate census data concerning numbers of leaves, tillers, etc., were made at frequent intervals. Plants showed signs of nitrogen deficiency about 90 days after sowing and additional nitrate (see above) was added. Inflorescences first appeared 114 days after sowing.

Harvests were made on the following days after sowing: Harvest I, 15 days; Harvest II, 27 days; Harvest III, 52 days; Harvest IV, 101 days; Harvest V, 140

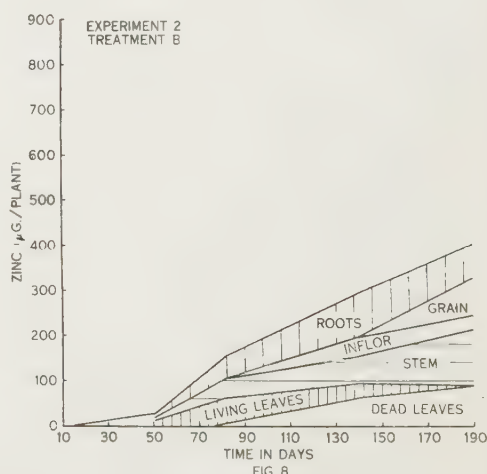
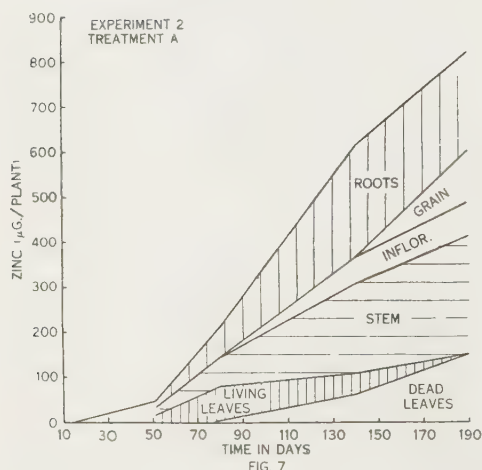
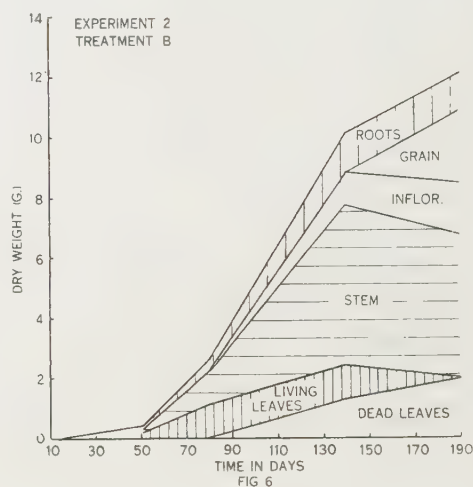
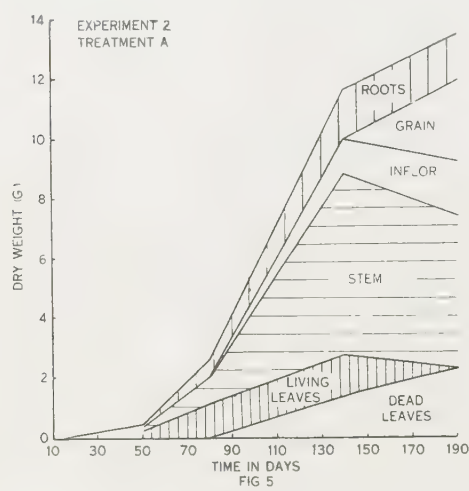
days; and Harvest VI, 190 days (maturity). At each harvest, plants were separated into appropriate parts, viz. living leaves, dead leaves, stem, root, inflorescence, and grain. After separation and before drying, roots were rinsed in dilute acetic acid and then in zinc-free distilled water in order to remove adsorbed zinc from the surface. All plant parts were rapidly dried under forced draught at 90°C. For each harvest, replicate material for analysis was drawn from the bulked samples of each treatment. Dry weights of plant organs per plant, plotted cumulatively, at different harvests are shown in Figures 1 and 2 and zinc contents in Figures 3 and 4. Percentage amounts of zinc are given in Table 1.

TABLE 1
RELATIVE AMOUNTS OF ZINC (MG. Zn PER KG. DRY MATTER)

	Harvest	I		II		III		IV		V		VI	
		A	B	A	B	A	B	A	B	A	B	A	B
Experiment 1	Living leaves	67	47	61	53	51	49	35	40	31	30	—	—
	Dead leaves	—	—	—	—	—	—	75	100	45	60	42	58
	Stem	—	—	—	—	51	66	39	25	20	17	21	19
	Inflorescence	—	—	—	—	—	—	—	—	35	37	18	15
	Grain	—	—	—	—	—	—	—	—	—	—	30	29
	Roots	550	420	237	269	308	169	71	97	106	53	34	32
	Whole plant	200	158	99	96	90	66	47	51	41	31	31	27
Experiment 2	Living leaves	58	58	68	54	61	48	39	27	—	—	—	—
	Dead leaves	—	—	—	—	318	317	47	49	69	46	—	—
	Stem	—	—	97	57	74	44	32	14	50	28	—	—
	Inflorescence	—	—	—	—	—	—	51	38	43	20	—	—
	Grain	—	—	—	—	—	—	—	—	43	33	—	—
	Roots	222	222	180	88	128	124	133	79	145	59	—	—
	Whole plant	159	159	95	60	82	61	53	29	60	33	—	—
Experiment 3	Living leaves	76	76	276	87	119	27	41	11	19	7	—	—
	Dead leaves	—	—	—	—	671	117	288	40	40	23	35	18
	Symptom leaves	—	—	—	—	—	—	—	14	—	5	—	—
	Stem	—	—	—	—	91	19	41	9	14	5	12	8
	Inflorescence	—	—	—	—	—	—	—	—	55	—	21	16
	Grain	—	—	—	—	—	—	—	—	—	—	33	16
	Roots	256	256	396	39	35	2	19	29	23	39	33	17
	Whole plant	164	164	305	73	105	20	50	14	19	11	21	12

Experiment 2.—Plants were grown in Laffer sand obtained from an area carrying only a sparse, natural grass covering. Cultural technique was similar to that described in Experiment 1, except that all pots received 0.004g. $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ initially plus 0.002g. H_3BO_3 ; $\text{Fe}_2(\text{SO}_4)_3$ was substituted for FeCl_3 , being prepared by oxidation of recrystallized FeSO_4 by Zn-free sulphuric and nitric acids. An additional 1.0g. NaNO_3 was added per pot 50 days after sowing. Pots of Treatment A received 0.08g. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (18,200 μg . Zn) per pot; pots of Treatment B received no additional zinc.

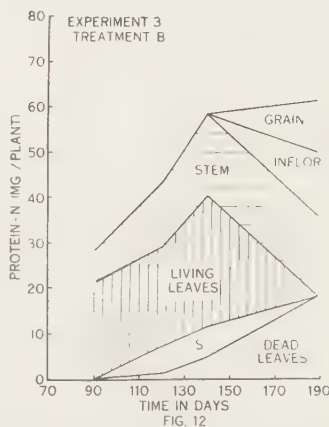
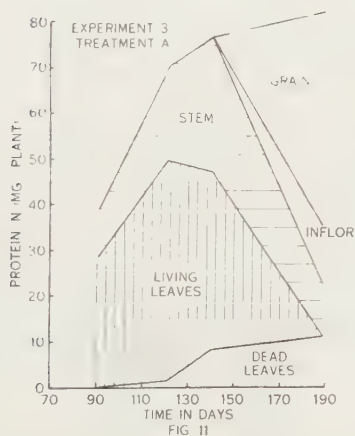
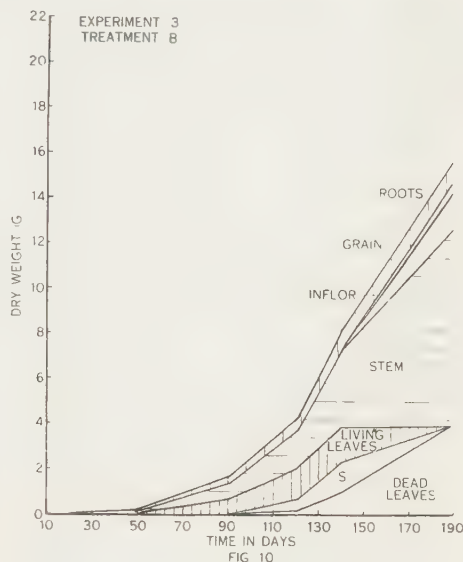
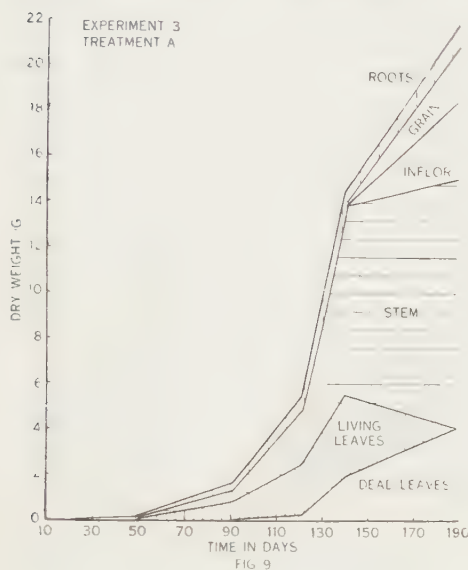
Seeds were sown on April 28, 1947 and inflorescences appeared 126 days after sowing. Harvests were made on the following days after sowing: Harvest I, 16 days; Harvest II, 51 days; Harvest III, 80 days; Harvest IV, 140 days; and Harvest V, 191 days (maturity). Separation of plant organs was made as described above. Dry weights of plant organs, plotted cumulatively, are shown in Figures 5 and 6, and their zinc contents in Figures 7 and 8. Percentage amounts of zinc are given in Table 1.



Figs. 5-8.—Dry weights (g. per plant) and absolute amounts of zinc in plant organs, Experiment 2.

Experiment 3.—Oat seedlings were germinated on waxed mosquito netting over glass-distilled water and when 16 days old were transferred to glass museum jars blackened externally, and each containing 2.5 litres of nutrient solution with the following composition (in g. per litre): KNO_3 , 1.0g.; KH_2PO_4 , 0.5g.; NaCl .

0.1g.; $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 0.5g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5g.; $\text{Fe}_2(\text{SO}_4)_3$, 0.017g.; H_3BO_3 , 0.5mg.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ equivalent to 0.5 mg. Mn.; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ equivalent to 0.1mg. Mo; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ equivalent to 0.5mg. Cu. The pH of the solution was adjusted to pH 5.2. Salts were purified as in previous experiments. Nutrient

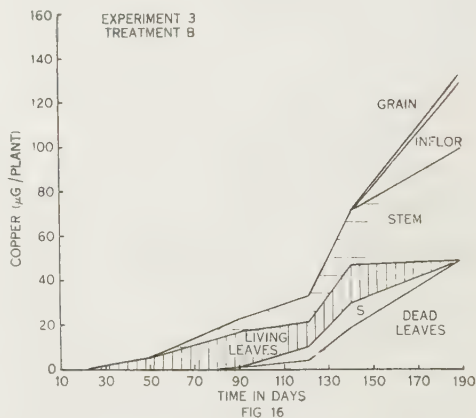
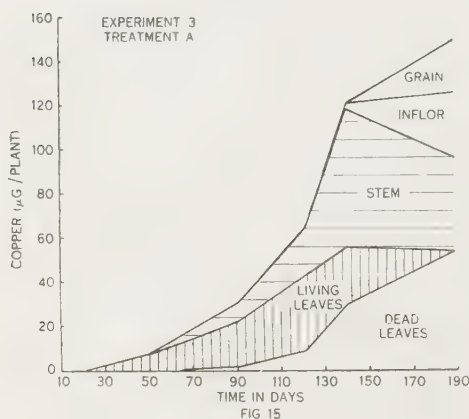
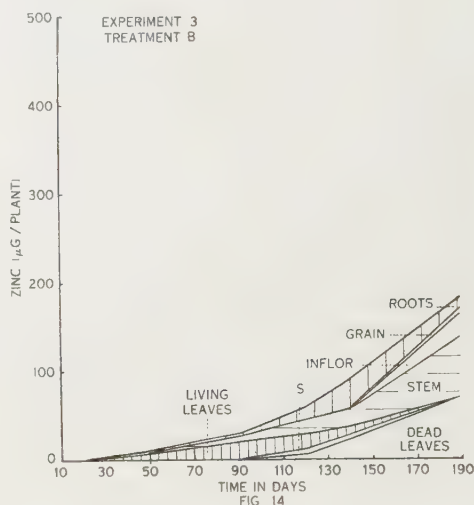
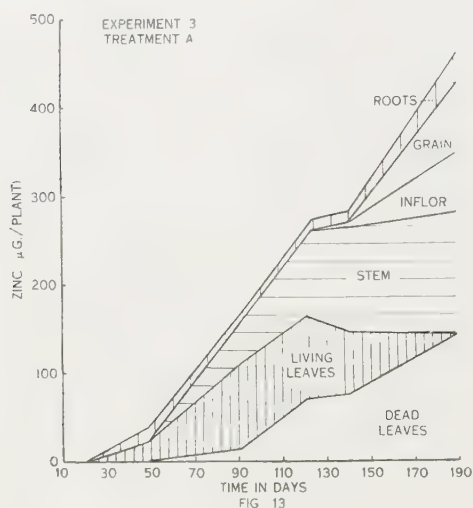


Figs. 9-12.—Dry weights (g. per plant) and absolute amounts of protein in plant organs, Experiment 3.

solutions were changed completely on days 58 and 88 and an additional 1.0g. KNO_3 per litre was added to all pots on day 73 and 0.5g. per litre on day 126. All nutrient solutions were aerated mechanically throughout the duration of the experiment. Six plants were grown in each jar.

Two treatments were applied: pots of Treatment A received 0.2mg. Zn per litre as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; pots of Treatment B received no additional zinc.

Seeds were germinated on May 17, 1948; lesions characteristic of zinc deficiency appeared on leaves of plants of Treatment B 95 days after sowing. These leaves were harvested and analysed separately. Inflorescences emerged in plants of Treatment A 146 days after sowing and in those of Treatment B 8 days later. Harvests were made on the following days after sowing: Harvest I, 21 days; Harvest II, 49 days; Harvest III, 91 days; Harvest IV, 121 days; Harvest V, 140 days; and Harvest VI, 189 days (maturity).



Figs. 13-16.—Absolute amounts of zinc and copper in plant organs, Experiment 3.

In this experiment, in addition to zinc contents, protein and copper contents of plant organs were determined. Dry weights of plant organs, plotted cumulatively, are shown in Figures 9 and 10; protein contents in Figures 11 and 12; zinc contents in Figures 13 and 14; and copper contents in Figures 15 and 16. Values for leaves exhibiting deficiency symptoms are labelled "S" in Figures 10, 12, 14, and 16. Percentage amounts of zinc are given in Table 1.

(b) Analytical Methods

Zinc and copper were determined polarographically in a Leeds and Northrup polarograph, using a nitrogen gas-chain. Wet digestion of one gram of dried plant material and subsequent procedures up to entry into the polarizing cell followed those employed by Walkley (1942). In Experiments 1 and 2, the basal solution used was similar to that used by Walkley, the quantity for each estimation being greater owing to the different size of cell used; in Experiment 3, the material prepared for polarization was dissolved in 2.5 ml. basal solution consisting of equal parts of 1N ammonia and 1N ammonium chloride solutions containing 0.02 per cent. gelatin; from this solution 2 ml. were placed in the electrolytic cell and both zinc and copper determined in successive steps on the polarogram. Standards for zinc and copper were run with each batch of five or six digestions. In the solution used, the zinc and cobalt steps coalesce, but the amount of cobalt present is negligible.

Protein-N was determined by the micro-Kjeldahl method after extraction of dried material and precipitation at pH 4.5 with trichloroacetic acid.

Chloroplasts were isolated and chlorophyll determined according to the methods described by Hanson, Barrien, and Wood (1941).

III. RESULTS AND DISCUSSION

(a) Total Zinc Content and its Relation to the Whole Plant

In all experiments the zinc content (in $\mu\text{g.}$ per plant) increased throughout the life cycle of the plant, indicating continuous uptake from the medium (Figs. 3, 4, 7, 8, 13, 14).

The absolute amount of zinc present in the whole plant at final harvest (approximately 190 days in each experiment) showed considerable variation between the different experiments.

In Experiment 3 the plants apparently absorbed the zinc completely from the culture solutions. In Treatment B each group of six plants at final harvest contained 1122 $\mu\text{g.}$ Zn absorbed from the culture solution and derived from contamination in distilled water, chemicals, and atmospheric dust; in Treatment A, 1500 $\mu\text{g.}$ Zn were added to the nutrient solution in each jar over the growing period and each group of six plants in this treatment contained 2700 $\mu\text{g.}$ Zn. This latter figure is approximately the sum of that in the untreated plants plus that supplied in solution. It would seem, therefore, that the efficient use by plants of relatively small quantities of zinc supplied in solution is analogous to that described for copper by Piper (1942).

On the other hand, in the experiments in the soil cultures it is clear that large amounts of zinc were not readily available to oat plants and were apparently immobilized in the soil. In Experiments 1 and 2, the Laffer sand contained 1 part per million of zinc, i.e. 3000 $\mu\text{g.}$ Zn were present in the soil in each pot.

In Experiment 1, 2300 $\mu\text{g.}$ Zn per pot were added to the sand in Treatment A, yet at final harvest the three plants in each pot of this treatment contained 678 $\mu\text{g.}$

Zn whilst that of the controls contained 585 $\mu\text{g.}$, i.e. the treated plants contained only 93 $\mu\text{g.}$ more than those untreated.

In Experiment 2, 18,200 $\mu\text{g.}$ Zn were added to the sand in Treatment A; the three plants in each pot at final harvest contained 2467 $\mu\text{g.}$ Zn whilst the three control plants contained 1206 $\mu\text{g.}$ Zn; i.e. the treated plants contained about twice as much zinc as the controls but this increase was small compared with the proportionate increase in amount of zinc added to the medium.

When treated plants contained much greater amounts of zinc at final harvest than the controls, as in Experiments 2 and 3, the increased zinc content was obvious at early harvests and was maintained throughout the growth cycle (see Figs. 7, 8, 13, 14).

It appears, therefore, that zinc uptake was increased when the amount of available zinc in the medium was increased, but in the Laffer sand much zinc was not readily available to the plant.

Differences in yield of dry matter between experiments in different years are probably due to the different seasons and difference in cultural conditions, and no comparison can be made on this basis.

Within any one experiment the total yield of dry matter of the whole plant and its parts at final harvest did not show significant differences (t test) between treated and untreated series, except in Experiment 3. In this case, characteristic zinc-deficiency symptoms appeared on the leaves and the yield of grain was greatly depressed.

In Experiment 1, treated plants contained only 31 $\mu\text{g.}$ Zn per plant more than the controls, the increase in zinc content occurring chiefly in the roots of the treated plants; there was no significant difference in dry weight at final harvest between treatments.

In Experiment 2 also there was no significant difference in dry weight at final harvest although the treated plants contained approximately twice as much zinc as the controls. This confirms the effects of "luxury" amounts of zinc which have been described by Reed (1942) for peas and beans, and by Lyon and Beeson (1947) for turnips and tomatoes.

Experiment 3 indicates clearly that there is a threshold value for zinc below which grain is not produced.

Zinc contents on a relative basis (mg. Zn per kg. dry matter) for the whole plant are shown for the three experiments in Table 1. In all cases this relative value for zinc decreased with increased age of the plant. This decrease was caused by exponential increase in dry matter associated with a decrease in rate of uptake owing to depletion of external supply. The small change in value of this ratio towards the end of the life cycle shown in all experiments suggests that, over this period, the amounts of dry matter produced may be determined by the amount of zinc absorbed.

The minimum amount of zinc required by an oat plant for full development depends upon its size; the oat plants in Experiments 2 and 3 (Treatment A) were average, well-developed plants and the data suggest that about 400 $\mu\text{g.}$ Zn per plant are necessary for full development. The data for Experiment 3 suggest

that deficiency symptoms and failure of grain formation occur when the concentration of zinc in the whole plant is less than 20 parts per million on a dry weight basis at a time when the inflorescence is developing rapidly and prior to its exertion.

(b) *Distribution of Zinc in Plant Organs*

The absolute amounts of zinc and also the dry weights of the whole plant and its component organs in each experiment are shown in Figures 1-14. The distribution of zinc in each organ as a percentage of the total zinc content is given in Figures 17-19.

Up to the time of penultimate harvest the absolute zinc content of all plant organs increased whilst the relative content (parts per million of dry matter) decreased. This period was one of active growth and the reasons for decrease in the ratio have been discussed.

Between penultimate and final harvests in all experiments the inflorescence and grain developed; the root system had attained its maximum weight at penultimate harvest and thereafter decreased owing to translocation of material to the developing inflorescence; no new leaves were produced during this period but already-formed leaves became senescent and material was transported from them. Transport of protein from leaves and stems in Experiment 3 during this period is shown in Figures 11 and 12. Differences between Treatments A and B in Experiment 3 are noteworthy in this respect for deficiency of zinc in Treatment B resulted in little grain formation, consequently these plants remained vegetative for a longer time than in Treatment A and the effect of the small "sink" caused by the grain is reflected in the smaller percentage loss of protein from the leaves and stems; in this experiment copper also was transported from the stem in Treatment A but not in Treatment B.

During this period, the demand for zinc by the developing inflorescence and grain must be met either by translocation from other organs or by absorption from the medium. It has been pointed out already that the relative amounts of zinc in the whole plant in each treatment showed little change between penultimate and final harvests. Between these harvests the zinc uptake for the whole plant showed high correlation ($r = 0.843$) with increase in dry weight of stems *plus* inflorescence *plus* grain over that period. It would appear, therefore, that during development of the inflorescence and grain much of the zinc is absorbed from the medium, though it is apparent from Figures 1-14 that some may be transported, especially from the roots. The amounts of zinc in individual organs and its distribution are discussed in detail below.

Leaves.—Usually the leaves contained from 20 to 30 per cent. of the total zinc in the plant over the greater part of the life cycle (*vide* Figs. 17-19). On a relative basis, the zinc content of the living leaves varied considerably with the cultural treatment but within any one treatment the ratio decreased in a regular manner throughout the life cycle (Table 1) and for the reasons discussed previously.

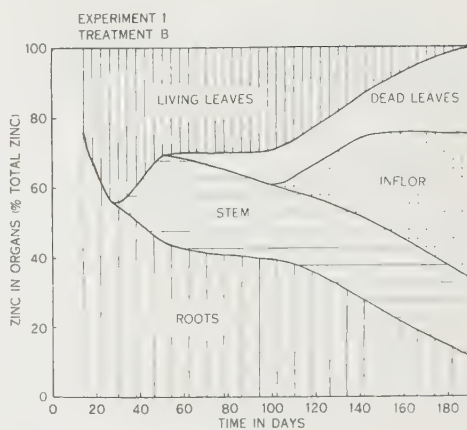
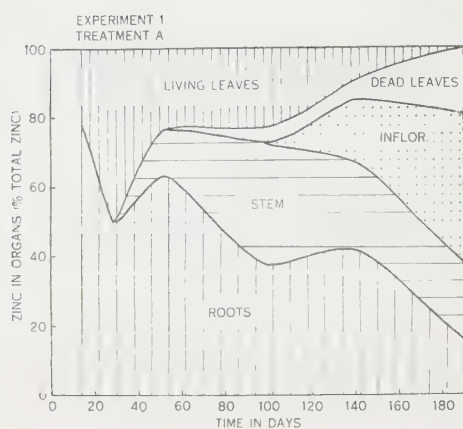


FIG 17

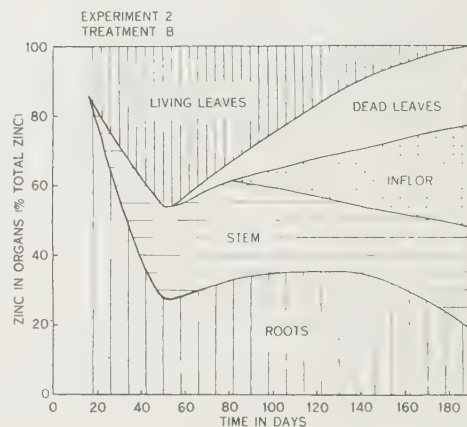
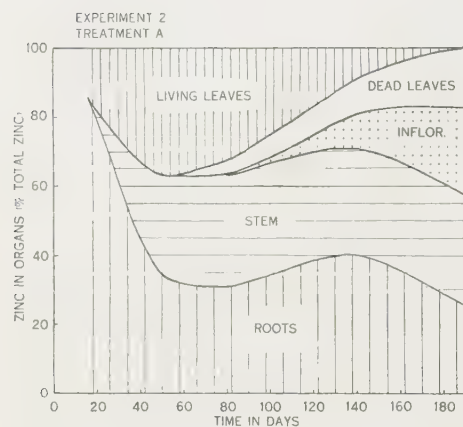


FIG 18

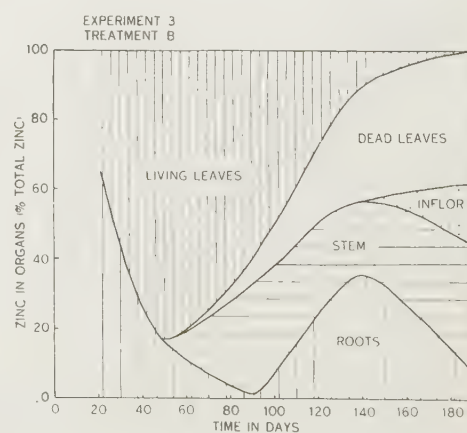
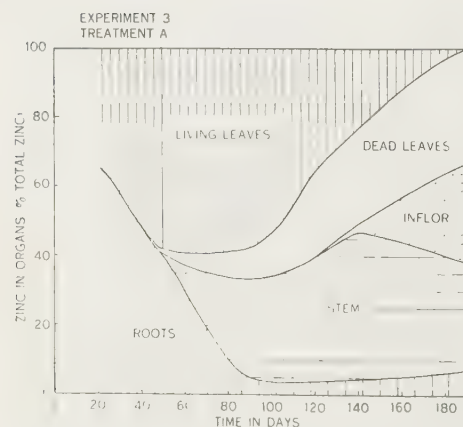


FIG 19

Figs. 17-19.—Amounts of zinc in plant organs as percentages of total zinc per plant, Experiments 1, 2, and 3.

The outstanding feature about the behaviour of zinc in leaves in these experiments is that, in every treatment, zinc was apparently immobilized in the leaves and was not transported to other organs as the leaves became senescent and died. This is shown in Figures 3, 4, 7, 8, 13, and 14 where it may be seen that the absolute zinc content of dead leaves at final harvest is equal to the sum of the living *plus* dead leaves at the penultimate harvest except in Treatment B, Experiment 3, where failure of grain formation permitted accumulation of zinc.

Up to the time of penultimate harvest it cannot be said with certainty that zinc was not transported from the leaves; the absolute amount of zinc increased up to this time but immobility is suggested by the high relative zinc contents of dead leaves. The relative zinc content of dead leaves was considerably higher than that of living leaves (Table 1) since the bases of expression were not comparable, dead leaves having lost protein as well as carbohydrates. Our experience in other experiments (Wood and Womersley 1946) has been that at the earlier harvests the dry weight of dead leaves was 50-70 per cent. of that of the dry weight of the same leaves when they were living.

In Experiment 3, leaves exhibiting symptoms of zinc deficiency were collected and analysed separately. The symptoms appeared on third and fourth leaves behind the growing point which were nearing maturity. The symptoms consisted of grey-fawn patches which appeared first on the upper parts of the leaves and the lesions then extended down the blade, purple colourings appearing as well. Chloroplast disintegration and lysis of cell contents occurred in these leaves. The final purple colour suggests connection of zinc deficiency with deranged phosphorus metabolism.

Whether translocation of zinc occurred from leaves under these circumstances is difficult to determine. Leaves exhibiting symptoms contained considerably less zinc at Harvest V in Experiment 3 than the whole sample of living leaves at that Harvest, but it must be remembered that the leaves exhibiting symptoms were late-formed and consequently would be expected to have lower zinc contents on a dry weight basis than those formed earlier which made up the bulk of the whole sample. Whilst, therefore, it cannot be said with certainty that no translocation of zinc occurred under these conditions, the data for absolute amounts of zinc indicate that if translocation occurred the amount must have been very small.

It appears, therefore, that in oat leaves zinc behaves in a similar manner to copper which Wood and Womersley (1946) have shown is not transported from leaves. The copper contents of plant organs in Experiment 3 confirm these findings (Figs. 15 and 16).

As might be expected from the different behaviour of zinc and protein in leaves (see Figs. 13, 14, 15, and 16) there was no simple relationship between these two variables. It is probable that zinc in leaves is bound with a colloidal constituent for zinc was not removed from leaves by dialysis. Fifty grams of oat leaves were macerated in a Waring Blendor for 3 minutes with

125 ml. iced water; the mass was centrifuged at a low speed sufficient to remove cell wall debris, etc. and of the supernatant suspension 15 ml. were dialysed in a Viskin membrane against distilled water for 24 hours. Zinc estimations showed that before dialysis the suspension contained 14.2 μ g. Zn and after dialysis 15.2 μ g. Zn, i.e. no zinc was removed by dialysis under these conditions.

The distribution of zinc within the leaf was further investigated by isolating pure whole chloroplasts according to the method described by Hanson, Barrien, and Wood (1941), determining the zinc, copper, and chlorophyll contents of the chloroplasts and comparing the amounts so obtained with those present in the original whole leaf material. The percentage of total leaf zinc present in the chloroplasts of oats in two separate determinations was 16 and 20 per cent. respectively; the amount of copper present in chloroplasts as a percentage of total copper was 35 and 40 per cent. respectively.

This work was extended by isolating chloroplasts from spinach (in which isolation is easier) and determining in a similar way zinc, copper, protein, and chlorophyll contents. In three separate estimations the percentage of total zinc occurring in the chloroplasts was 51, 50, and 53 per cent. respectively. In the last two estimations protein-N and copper contents were also determined; the chloroplasts contained 40 and 41 per cent. total leaf protein-N and 45 and 42 per cent. respectively of the total leaf copper.

It is apparent, therefore, that part of the leaf zinc was localized in the chloroplast although it would seem that variations in the proportion so localized occur between species—only about one-fifth of the total leaf zinc being present in the chloroplasts in oats and about one-half of the total leaf zinc in spinach. This distribution is in contrast with that of copper where there was little difference between the two species in percentage distribution between chloroplasts and whole leaf.

Grain.—The zinc contents of grain on a dry weight basis are given in Table 1; they reflect the zinc status of the whole plant at maturity, being most marked in the small amount of grain produced by plants in Treatment B in Experiment 3 which exhibited zinc-deficiency symptoms in leaves. The mean value for oat grains from a number of experiments, some not recorded here, in which zinc-deficiency lesions were not evident was found to be 35 μ g. Zn per g. of dry weight of grain. The developing grain represents a “sink” for zinc; since zinc was not transported from leaves it is apparent that the source of the zinc must be stems, roots, or absorption from the medium.

Stems and Inflorescence.—The absolute zinc content of stem *plus* inflorescence increased throughout the life cycle except in Experiment 1, where little change occurred between penultimate and final harvests. In these experiments, therefore, there was no evidence of translocation from the stem—from the data of Experiment 3 (Figs. 11-14) it is clear that the stem was losing protein whilst accumulating zinc.

Roots.—The absolute amount of zinc in the roots increased up to the penultimate harvest and thereafter decreased up to the time of final harvest. Decrease in amount of zinc between these two harvests was correlated with

decrease in dry weight of the roots over the same period ($r = 0.826$). The zinc apparently moved from the root along with other metabolites.

The absolute amounts of zinc in the roots varied considerably in the different experiments, being noticeably low in the poorly developed root systems of plants in the water culture (Experiment 3). In other experiments, with a different nutrient regime (to be described elsewhere) the authors have found relatively large absorption of zinc by the roots with considerable loss between penultimate and ultimate harvests, the general picture conforming to that shown in Figure 3.

The greater proportion of total zinc found in the roots of Treatment B compared with that found in Treatment A in Experiment 3 is possibly the result of renewal of the culture solutions on day 88 associated with partial failure of flower formation at that time.

Summing up these studies, it is apparent that zinc is absorbed continuously throughout the life cycle of oat plants and redistribution of zinc occurs during the development of grain and inflorescence. No zinc is translocated from leaves to other organs but zinc is supplied to inflorescence and grain from the root and from the medium. Piper and Walkley (1947) have stated that zinc is translocated to the grain from straw as maturity approaches. However, this conclusion was reached from consideration of the percentage distribution of zinc between grain and straw at final harvest. Such a deduction in fact cannot be made from relative figures at final harvest, but only from absolute contents per plant determined at intervals throughout the life cycle.

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STUDIES ON THE NITRATE REDUCTASE OF *ESCHERICHIA COLI* IN THE CELL-FREE STATE

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Summary

A method of purifying the nitrate reductase obtained by crushing *Escherichia coli* cells in a ground glass mill is described, involving elution at pH 9.5 and precipitation into slightly acidified acetone, followed by treatment with phosphate buffer (pH 8.0) and dialysis. Methods of estimating the activity of the enzyme without recourse to coupling with other enzymes are outlined, viz.: (a) the oxidation time method; (b) the use of photochemically reduced methylene blue as hydrogen donor.

The action of various inhibitors on the enzyme was studied. The following significant results emerge:

(a) The enzyme is strongly inhibited by cyanide and azide.

(b) The enzyme is largely unaffected by reagents for iron and by carbon monoxide.

(c) The enzyme is unaffected by the majority of the so-called -SH reagents, but is inhibited by heavy metal ions; however, this is most probably due to their action of precipitating the enzyme, as used here, in extremely small concentrations.

(d) Nitrophenols inhibit the reduction of nitrate in the cell-free state.

The reaction between hydrogenase and nitrate reductase depends on the presence of a certain carrier present in boiled bacterial suspensions and replaceable by Nile blue and flavoprotein. Evidence for the existence of a separate enzyme, nitrite reductase, is presented. The nature and physiological significance of nitrate reductase are discussed in the light of the above findings.

I. INTRODUCTION

That nitrate can supplant the function of oxygen and act as a hydrogen acceptor has been known for a long time (e.g. Niklewski 1914; Ruhland 1924); nor is nitrate unique in this respect among inorganic ions, for nitrite, chlorate, sulphate, sulphite, and others, likewise accept hydrogen. Quastel and Woolridge (1929) were the first to show that the reduction of nitrate was enzymic, for until then it had been thought that nitrate acted rather like methylene blue (MB). It was noted quite early that even such closely related ions as nitrate and nitrite, or sulphate and thiosulphate, are not reduced by the same enzymes (Quastel, Stephenson, and Whatham 1925; Yamagata and Nakamura 1938) for there exist bacteria which reduce only one of each of these pairs of ions.

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Stickland (1931), studying the nitrate-reducing enzyme of *Escherichia coli*, found it to be uninhibited by carbon monoxide, not affected by toluene treatment, but completely inhibited by cyanide. He found that the "nitrate reductase" could be coupled with many dehydrogenases which are thereby enabled to oxidize their substrates under anaerobic conditions. Aubel and Egami (1936) were able to isolate an organism which oxidatively de-aminated *l*-alanine under anaerobic conditions using nitrate, and nitrate only, as the hydrogen acceptor.

Woods (1938) studied the reduction of nitrate to ammonia by means of molecular hydrogen in *Clostridium welchii*. Many other bacteria failed to do this, however, and only reduced nitrate to nitrite. The latter compound can easily be shown to be an intermediate in the reduction of nitrate to ammonia, but the further steps are obscure though Aubel (1938*a*, 1938*b*) has claimed that hydroxylamine and hyponitrous acid are intermediates.

Lascelles and Still (1946) studied the system hydrogenase-nitrate reductase in *E. coli*. This organism reduced nitrate at a rapid rate, but nitrite was reduced only if a redox dye such as benzyl viologen was added; evidently hydrogenase and "nitrate reductase" needed a special carrier. They found that nitrate reduction was inhibited by cyanide and azide.

Very little work has been done on nitrate reductase in the cell-free state. Lemoigne, Desveaux, and Gavard (1944) claimed that the nitrate reductase of *E. coli* consists of an enzyme complex, partly in the culture fluid and partly in the cell; either factor alone is inactive. Yamagata (1938*a*, 1938*b*), however, remains the most important contributor. He obtained a cell-free preparation of nitrate reductase from *E. coli* by autolysis at 30°C. The enzyme was adsorbed on Berkefeld filters, failed to oxidize reduced coenzyme I and was strongly inhibited by 0.01M cyanide.

The present work was undertaken with a view to preparing a cell-free preparation of nitrate reductase by crushing cells of *E. coli* in a ground glass mill, purifying, and if possible, isolating the enzyme, and to study the effect of inhibitors on it in order to expose any prosthetic group.

II. EXPERIMENTAL METHODS

The strain of bacteria used in this investigation was strain B of *E. coli*, kindly supplied by Dr. M. Delbrück. The method of growing, collecting, and crushing the bacteria has been outlined in a previous communication (Joklik 1950). In order to increase the yield of nitrate reductase the addition of nitrate to the medium was tried, but no significant increases in nitrate reductase content were observed.

Among the various chemicals used in this study which were prepared in the laboratory were the following:

(*a*) *Kojic acid*.—A strain of *Aspergillus flavus oryzae*, kindly supplied by Mr. J. M. Vincent of the School of Agriculture, University of Sydney, was grown at 37°C. under the most aerobic conditions possible, oxygen being con-

tinually passed through the medium. After three weeks the mycelium was filtered off and the mother liquor evaporated till crystals formed. In general, the procedure of Raistrick *et al.* (1931) was followed but not all the purification steps were carried out.

(b) *Flavoprotein* was prepared following the method of Straub (1939) to the adsorption on alumina Cγ.

(c) *Coenzyme I* was prepared according to the method of Umbreit, Burris, and Stauffer (1945).

(d) *Carbon monoxide* was prepared by the action of concentrated sulphuric acid on formic acid. The gas was passed through three traps containing respectively alkaline sodium hydrosulphite, alkaline pyrogallol, and concentrated sulphuric acid.

(e) *Janus red* was prepared according to the method of Fischer and Eysenbach (1937) except that Janus green B was used as the starting compound.

Nitrite was estimated with the Griess-Ilosvay reagent. Since the concentration of nitrite formed was usually very great, the practice adopted was to dilute the sample 1 in 150, whereby removal of any dye or deproteinization became unnecessary. Where, however, deproteinization was necessary, this was done by adding to the reaction mixture half a volume of glacial acetic acid and one-sixth the volume of saturated ammonium sulphate, and placing in a boiling water-bath. Within one to two minutes the proteins had usually flocculated completely, and were filtered off through a Whatman No. 41 filter paper. This procedure had to be worked out since all the other common protein precipitating reagents, even trichloroacetic acid, interfered with the estimation of nitrite.

Estimation of the colour developed was carried out by means of a photoelectric absorptiometer, using a green filter. Standard curves were constructed frequently.

III. ESTIMATION OF THE ENZYME

Many workers have estimated the rate of reduction of nitrate in tissues, but the method has always been to couple nitrate reductase with a dehydrogenase, and to estimate the nitrite produced. Questel, Stephenson, and Whetham (1925), for instance, used enzymically reduced leucomethylene blue as a hydrogen donor, so that the rate of re-oxidation in the presence of nitrate and nitrate reductase represented the difference of the activities of nitrate reductase and the dehydrogenase. Fischer and Eysenbach (1937), however, estimated fumaric hydrogenase by reducing a dye (Janus red) with a slight excess of sodium hydrosulphite, which was then oxidized away so that they were dealing with a known amount of non-enzymic hydrogen donor.

In the present study, nitrate reductase was so active that the small volume of very dilute (0.0001M) hydrogen donor used by Fischer and Eysenbach would have been quite inadequate; hence larger quantities of sodium hydro-

sulphite had to be used. That substance acts as the donor, while the dye (Janus red), of which a very small amount suffices, acts as the carrier. It can be shown that the rate of the reaction is a function of dye concentration only when this is less than 0.00005M. The creation of anaerobic conditions presented a problem since sodium hydrosulphite decomposes in an unpredictable way *in vacuo*, depending on the surface of the glass. Anaerobic conditions were therefore maintained by placing a layer of paraffin oil about two centimetres thick over the reaction medium. By this means a given sample of reduced dye was easily kept in the reduced state for periods from two to six days; it was perfectly satisfactory for experiments lasting at the most one hour. Aubel, Schwartzkopf, and Glaser (1937) have suggested that nitrite could re-oxidize leucodyes chemically. No evidence for this was found under the conditions used here; it is probable that the amounts of nitrite formed were too small to cause such an effect.

The general procedure was to place into a test-tube 0.1M Sørensen phosphate buffer (pH 7.6), enzyme, nitrate (equal to the amount of sodium hydrosulphite used), and sodium hydrosulphite (0.3 to 0.8 ml. of a 0.1M solution, the amount being chosen so that the time the dye takes to change colour (the oxidation time) is between six and twenty minutes). The tube was then incubated in a water-bath at 37°C. and left to attain the temperature. Any inhibitors were now added. Within four minutes after placing the tubes in the water-bath (for if left longer the sodium hydrosulphite decomposes) 1.0 ml. of a 0.0004M solution of Janus red (JR) or other oxidation reduction indicator (ORI) and 2.0 ml. paraffin oil were quickly added and the time noted on a stop watch. The tube was then gently agitated from time to time by rotating between the fingers, and the time when the ORI changed colour noted to the nearest five seconds. Duplicates done at the same time agreed to within 2 per cent.; in separate runs, however, oxidation times may differ by 5 per cent. since the sodium hydrosulphite decomposes rapidly at 37°C. in aqueous solution, as shown in Table 1.

TABLE 1
DECOMPOSITION OF SODIUM HYDROSULPHITE AT 37°C. IN AQUEOUS SOLUTION*

Time of Addition of JR and Paraffin (min.)	Oxidation Time (min. & sec.)
0	15-00
10	12-00
20	10-20
30	8-15
40	6-50

* Each tube contained 2.0 ml. buffer, 0.2 ml. of enzyme solution, 0.4 ml. 0.1M KNO_3 , and 0.8 ml. $\text{Na}_2\text{S}_2\text{O}_4$. JR and paraffin were added to the first tube at time 0, and then 10, 20, 30, and 40 min. later to the other ones. The oxidation times were noted in each case.

When no enzyme was added to the tubes, the ORI stayed in the reduced condition for over 24 hours, since, even though all sodium hydrosulphite might

have long been decomposed, the ORI will not become re-oxidized because no oxidizing agent is present. Similarly, if small amounts of enzyme are used, the ORI will remain reduced for a long time, since only very little sodium hydrosulphite will be used up by it. Readings were rarely continued for more than 60 minutes.

When the nature or colour of a reagent used rendered clear observation of the end-point by eye impossible, the nitrite formed was estimated. It was found that, with the amount of sodium hydrosulphite generally used, 0.1 ml. of the reaction mixture mixed with 15.0 ml. of distilled water gave, after addition of the Griess-Ilosvay reagent, a colour which could be read accurately on the instrument used. This large dilution made it unnecessary to either deproteinize or to remove the ORI; it also stopped the reaction immediately. It is necessary, when estimating nitrite after a certain time, to have excess reducing agent still present; otherwise nitrite production has stopped and the amount of nitrite formed under these conditions is no measure of the activity of the enzyme. However, the amount of sodium hydrosulphite left must not be large, otherwise the pink diazo compound is not formed. In practice the dilution of 1 : 150 depresses the concentration of hydrosulphite sufficiently to permit the colour to develop normally. After 20 minutes the colours were read on the instrument, and referred to a standard curve. The results were usually expressed in micrograms nitrite/ml. of the solution, the colour of which was measured; to express this figure as mg. of nitrite formed in the reaction mixture, this would have to be multiplied by $0.17x$, where x is the volume of the reaction mixture in ml.

Experiments were carried out to test whether the amount of nitrite formed was actually related to the amount of sodium hydrosulphite used and whether the reaction stopped completely after the ORI had been re-oxidized. The relative efficiencies of JR, benzyl viologen (BV), and methylene blue (MB) were also estimated by comparing the time which elapsed before they changed colour (Table 2).

Table 2 indicates that the nitrite concentration is apparently a linear function of the time of incubation, that the reaction stops when the ORI changes colour, and that the amount of nitrite produced is proportional to the amount of sodium hydrosulphite used. Similar experiments confirmed the fact that the reaction with BV as the carrier is quicker than that with MB.

One other method was used in the estimation of the activity of nitrate reductase. Yamagata (1938*a*) had suggested that the photochemical reduction of MB by kojic acid in strong light could be used for this purpose: a mixture of the correct amounts of the two compounds in buffered aqueous solution and illuminated with two 200-watt globes results in the reduction of the MB within 15 minutes.

In order to carry out these experiments, enzyme, buffer, nitrate, and inhibitor were pipetted into the lower part of a Thunberg tube, while into the stopper were placed the MB and kojic acid solutions. The tube was then evacuated and filled with nitrogen, the stopper illuminated till the MB was

reduced, and the tube placed in a water-bath and tipped. The rate of re-colouration was observed.

Owing to the high activity of the enzyme used here, a strong solution of MB had to be used. This results in the precipitation of the reduced MB; and the intense colours involved made impracticable their removal for the colorimetric estimation of nitrite formed. Yamagata used the method only to demonstrate the re-oxidation of reduced ORI; in the work described in this paper the method was used to observe the action of several inhibitors which were reduced by sodium hydrosulphite and to confirm results obtained with other methods. The results, however, were not quantitative.

TABLE 2
EFFECT OF SODIUM HYDROSULPHITE AND NITRATE ON THE EXTENT OF
NITRITE FORMATION BY NITRATE REDUCTASE*

$\text{Na}_2\text{S}_2\text{O}_4$ (ml.)	KNO_3 (ml.)	ORI	Time of Incubation (min.)	Oxidation Time (min. & sec.)	Nitrite Formed ($\mu\text{g.}/\text{ml.}$)
0.6	0.2	MB	10	—	0.27
0.6	0.2	MB	20	—	0.56
0.6	0.2	MB	30	—	0.95
0.6	0.2	MB	40	38-25	1.33
0.6	0.2	MB	50	37-50	1.44
0.6	0.2	MB	60	37-40	1.46
0.6	0.2	BV	30	24-10	1.20
0.2	0.1	MB	30	12-45	0.39
0.2	0.3	MB	30	11-55	0.33
0.4	0.2	MB	30	23-45	0.79
0.6	0.1	MB	30	—	1.16
0.6	0.3	MB	30	—	1.08

* Each tube contained 2.0 ml. buffer (pH 7.6), 0.5 ml. enzyme solution, 1.0 ml. of the ORI indicated, the specified amount of 0.1M sodium hydrosulphite, and 0.1M nitrate.

IV. PURIFICATION

The source of the enzyme was the organism *E. coli*, which had been ground in a ground glass mill according to the method outlined previously (Joklik 1950). When the bacteria had been crushed two or three times in distilled water and the extract centrifuged at 4500 r.p.m. for 20 to 25 minutes, most of the nitrate reductase was found to be in the insoluble residue (either still on major particles of the cell wall or on smaller submicroscopic particles) while the rest was either in solution or in such a finely divided state that centrifuging at the above-mentioned speed failed to deposit it. This latter fraction was largely precipitated by one-twelfth saturated ammonium sulphate; concentrations higher than half saturated were not tolerated by the enzyme.

Both the enzyme precipitated by ammonium sulphate and that adhering to the larger cell fragments could be brought into solution by dialysis against

borate buffer (pH 9.6) at 5°C. for 24 hours. The supernatant, which was slightly opalescent, was then added to six volumes of dioxane at 6°C., just above its freezing point. The precipitate should not flocculate and was centrifuged quickly; the dioxane was then drained off the precipitate, which was dissolved in 0.2M sodium phosphate (dibasic). After standing overnight, the precipitate was centrifuged down, and the nitrate reductase remained in solution. Acetone could be substituted for dioxane, and better results were sometimes obtained if the organic solvent was acidified slightly (about 10^{-5} M with regard to hydrochloric acid); it must be emphasized that, though the method has worked well and given active, soluble nitrate reductase, sometimes the precipitate after acetone or dioxane precipitation has been inactive. Probably all the factors involved in that precipitation are not yet known. A further step in the purification would be to concentrate the enzyme by freeze-drying, for, although, in the preparation described above, the enzyme was present in a perfectly soluble state and free from other enzymes (especially hydrogenase and nitrite reductase) except catalase, the actual amount present was relatively small. Freeze-drying did not affect the enzyme when unpurified in the bacterial extract; however, this process was not carried out, since the ordinary preparations were quite satisfactory for the work at hand.

V. PROPERTIES

(a) *Behaviour on Heating*

The enzyme is sensitive to heat. When two ml. portions of enzyme solution were plunged into a boiling water-bath and heated to different temperatures for 50 to 60 seconds, a temperature of 70°C. caused a loss in activity of almost 50 per cent., while at 80°C. the loss was over 75 per cent. (Table 3).

TABLE 3
EFFECT OF HEAT ON NITRATE REDUCTASE ACTIVITY*

Temperature attained (°C.)	20	50	60	70	80	90	95
Oxidation time (min. & sec.)	9-30	9-55	13-15	16-30	40-00	>60	>60

* Each tube contained 1.0 ml. enzyme solution, 1.0 ml. buffer (pH 7.6), 0.3 ml. nitrate (0.1M), 0.3 ml. $\text{Na}_2\text{S}_2\text{O}_4$ (0.1M), 1.0 ml. JR (0.0004M), and 2.0 ml. paraffin oil.

The heat treatment adopted for hydrogenase (Joklik 1950) would thus destroy most of the nitrate reductase.

(b) *Dilution*

No dilution effect could be demonstrated. That and the fact that nitrate reductase is not inactivated by dialysis make it probable that leuco-Janus red acts directly as the hydrogen donor without an intermediate carrier.

(c) *Substances Shortening the Oxidation Time*

It was noticed frequently that acetone seemed to have an accelerating effect on the enzyme activity; that is, the oxidation time was greatly reduced

TABLE 4
EFFECT OF ACETONE ON THE OXIDATION TIME*

Amount of Acetone (ml.)	Nil	0.1	0.2	0.3	0.4
Oxidation time (min. & sec.)	11-30	5-00	1-05	str.†	str.†

* Each tube contained 0.5 ml. enzyme solution, 2.0 ml. buffer, 0.2 ml. KNO_3 (0.2M), 0.4 ml. sodium hydrosulphite (0.1M), 1.0 ml. JR, and the specified amount of acetone.

† str. denotes that the JR remained in the oxidized state.

(Table 4). These results could be repeated even when partially heat-denatured enzyme was used. If, however, no nitrate was present, the ORI stayed in the reduced form in the presence of acetone as well as in its absence. It was clear, therefore, that the effect depended either on the amount of enzyme or on the amount of sodium hydrosulphite present; for these are the two factors controlling the oxidation time. The latter was found to be responsible, since evidence was obtained that, in the presence of a large excess of acetone (and even 0.1 ml. of acetone in 4.0 ml. would constitute a large excess), sodium hydrosulphite is destroyed, probably in the reduction of acetone. Sodium selenite and very small amounts of ferrous sulphate acted similarly; that is, the oxidation time was again greatly decreased (Table 5).

TABLE 5
EFFECT OF FERROUS SULPHATE AND SODIUM SELENITE ON THE OXIDATION TIME*

Concentration of Ferrous Sulphate (molarity)	Concentration of Sodium Selenite (molarity)	Oxidation Time (min. & sec.)
Nil	—	15-45
0.001	—	12-00
0.0005	—	9-00
0.00033	—	8-15
0.0002	—	9-15
0.0001	—	10-00
Nil	0.002	4-30

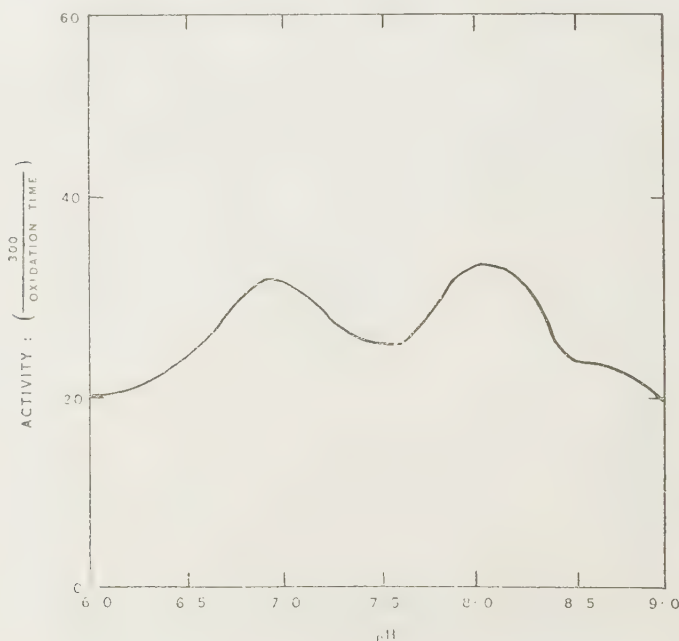
* Each tube contained 0.5 ml. enzyme solution, 2.0 ml. buffer, 0.2 ml. nitrate (0.2M), 0.4 ml. sodium hydrosulphite (0.1M), 1.0 ml. JR, and the specified amount of ferrous sulphate or sodium selenite.

These results are difficult to explain as sodium hydrosulphite could not have been used up in the reduction of the reagent added. Estimations done after experiments in which acetone, ferrous ions, and sodium selenite had been

used, revealed that only very small amounts of nitrite were formed; the reaction appeared to have stopped even before the ORI had changed colour. This would be caused, as has been discussed above, by the destruction of sodium hydrosulphite; by its oxidation in the presence of acetone, and, possibly, sodium selenite; and probably by a catalytic effect of very small concentrations of iron salts. However, references to none of these effects could be found in the common text-books of chemistry, nor does Beilstein mention sodium hydrosulphite as a possible reducing agent for acetone.

(d) *Effect of Acidified Acetone on the Enzyme*

In an attempt to split a prosthetic group from nitrate reductase, partially purified preparations were added to acetone containing from 10^{-6}M to 10^{-1}M hydrochloric acid. The precipitate was in each case centrifuged down, drained well, and re-suspended in phosphate buffer (pH 8.0). It was shown that this treatment had little effect on the enzyme provided conditions were kept as cool as possible till the concentration of hydrochloric acid reached 10^{-2}M , when complete inactivation resulted. Attempts to resynthesize the enzyme using inactive apoenzyme and haem failed.



(e) *Relation of Nitrate Reductase Activity to pH*

A pH curve over a wide range was constructed; however, it was impossible to proceed far into the acid region, since sodium hydrosulphite decomposes very readily at pH values below 5.5. Three different series of buffers were

used: citric acid-phosphate between pH 5.2 and 7.2; phosphate between pH 5.5 and 8.0; and glycine-sodium hydroxide between pH 8.0 and 10.0. The enzyme has two pH maxima, at pH 6.9 and 8.0 (Fig. 1).

(f) *Effect of Inhibitors*

(i) *Reagents for Iron- and Iron Porphyrin-containing Enzymes.*—The only reagents in this group which inhibited nitrate reductase were cyanide (inhibition 100 per cent. in 0.01M and 80 per cent. in 0.001M KCN); azide (100 per cent. inhibition in 0.01M solution); and potassium sulphocyanide (40 per cent. in 0.003M solution), the effect of which, however, is not due to its reaction with iron, since all iron was present in the reduced state. Among other substances tried and found to be without effect were hydrazine and hydroxylamine, thioglycolic acid, 8-hydroxyquinoline, α , α' -dipyrridyl, *o*-phenanthroline, and, as a general heavy metal reagent, diethyldithiocarbamate; none of these reagents caused any change in the oxidation time in concentrations ranging from M/100 to M/2000.

TABLE 6
EFFECT OF -SH REAGENTS AND HEAVY METALS ON NITRATE REDUCTASE

Inhibitor	Concentration (molarity)	Precipitation	Inhibition (%)
<i>o</i> -Mercuribenzoate	0.0025	—	90
<i>o</i> -Iodosobenzoate	0.0025	—	20
Benzamide	0.0025	—	20
Iodine	0.001	—	66
Copper sulphate	0.001	—	50 (?)
Mercuric sulphate	0.001	—	—
Cobalt sulphate	0.001	—	40
Chromic sulphate	0.002	+	40
Chromic sulphate	0.001	—	—
Ceric sulphate	0.002	+	50
Ceric sulphate	0.001	±	20
Ceric sulphate	0.0005	—	—
Stannous chloride	0.001	±	40
Stannous chloride	0.0005	—	10
Cadmium chloride	0.0002	+	30

(ii) *Reagents Specific for -SH Groups and Heavy Metal Ions.*—While investigating the effects of these two groups of inhibitors two difficulties were encountered. The first was that certain of the compounds tried were reduced by sodium hydrosulphite (e.g. *o*-mercuribenzoate, mercuric sulphate). The second was that most metal ions (cerium, cadmium, copper, iron, etc.) precipitated the enzyme in concentrations above M/500 and thereby caused inhibition. Whether highly purified enzyme would also be precipitated is not certain, as the enzyme preparation used in these experiments was not one of the purest obtainable by the method outlined above. However, the concentrations of metal ions used were low as it was assumed that if the metal

combined with -SH groups, or otherwise inhibited the enzyme to a significant degree, the effect would be noticeable at 0.001M concentration. Table 6 shows the results obtained.

Iodine and *o*-mercuribenzoate are seen to be the most important of the -SH inhibitors for nitrite reductase, but all inhibit to a certain extent. Several of the results set out in Table 6 were checked by estimating the nitrite formed (Table 7).

TABLE 7
EFFECT OF CERTAIN INHIBITORS ON THE FORMATION OF NITRITE

Inhibitor	Concentration (molarity)	Nitrite Found ($\mu\text{g./ml.}$)	Inhibition (%)
None	—	0.90	—
Mercuric sulphate	0.002	0.45	50
Ceric sulphate	0.002	—	100
Copper sulphate	0.002	0.10	90
Stannous chloride	0.002	0.94	—
Silver nitrate	0.002	0.73	30
Potassium ferrocyanide	0.0045	0.40	55
Potassium sulphocyanide	0.002	—	100
Sodium pyrophosphate	0.003	0.86	—
<i>o</i> -Mercuribenzoate	0.002	0.10	90
Phenyl mercuric acetate	0.002	0.30	66
<i>o</i> -Iodosobenzoate	0.002	0.86	—
α α' -Dipyridyl	0.002	—	100
Saturated toluene	—	0.87	—

Discrepancies were found for mercuric sulphate which caused a 50 per cent. inhibition of nitrite accumulation, and copper sulphate, which seemed to form a complex with JR and almost completely inhibited nitrite formation; likewise α α' -dipyridyl and potassium sulphocyanide here caused total inhibition (cf. Section (f) (i)).

The effect of carbon monoxide and oxygen was tested also, but neither gas had any effect on the enzyme. Alcohol and toluene did not affect the activity, while capryl alcohol and dioxane had an effect similar to that of acetone, though to a much smaller degree.

(g) Donor and Carrier Specificity

A series of experiments was carried out to determine whether nitrate reductase can use carriers other than reversibly reducible dyes, such as JR, MB, or BV. Among likely substances were those capable of being oxidized and reduced easily; but ferrocyanide, iron salts, cysteine, and ceric ions were all inactive.

Coenzyme I, flavoprotein, and cytochrome C were also used, but the amount of nitrite formed in their presence was no larger than in the absence of any carrier whatsoever; nor did these substances increase the rate of reaction when they were used in the presence of MB.

(h) *Estimation of Nitrate Reductase Activity with Photochemically Reduced MB*

The limitations of this method have already been outlined, as also the method of carrying out the experiments. It was used mainly for three reasons: to study the effect of substances reduced by sodium hydrosulphite, to determine whether nitrophenols are themselves reduced by nitrate reductase, and to detect any nitrite reductase present in the bacterial extracts. The last point will be commented on later. No evidence was obtained that nitrophenols themselves are reduced. The results with a variety of inhibitors are given in Table 8.

TABLE 8
EFFECT OF INHIBITORS ON NITRATE REDUCTASE, AS DETERMINED BY THE
RE-OXIDATION OF PHOTOCHEMICALLY REDUCED MB*

Inhibitor	Concentration (molarity)	Comment
None	—	The colour changes from light green to dark blue
Cyanide	0.002	} The colour stayed light green; full inhibition
Azide	0.002	
Mercuric sulphate	0.002	} No inhibition
Phenyl mercuric acetate	0.002	
<i>o</i> -Iodosobenzoate	0.002	
<i>o</i> -Mercuribenzoate	0.002	Greatly retarded; 90% inhibition
Sodium selenite	0.002	No inhibition
Silver nitrate	0.002	Colour changed to and stayed dark green; inhibition
Stannous chloride	0.002	} Slight inhibition
Urethane	1.0	
2,4-Dinitrophenol	0.002	} Strong inhibition
2,4-Dinitrophenylhydrazine	0.002	
<i>m</i> -Nitrophenol	0.002	} Inhibition 70% (?)
<i>p</i> -Nitrophenol	0.002	

* Each Thunberg tube contained from between 0.2 to 0.5 ml. enzyme solution, 2.0 ml. buffer (pH 7.6), and 0.3 ml. KNO_3 (0.1M), while the stoppers contained 0.5 ml. MB (0.2%), and 0.5 ml. kojic acid solution. The gas used was nitrogen; illumination was provided by three 150-watt globes for 20 minutes.

It appears from Table 8 that sometimes this method of estimating nitrate reductase activity gave results different from those obtained with other methods; especially was this so for mercuric sulphate, which showed no inhibition by this method. Possibly mercury ions, even though present only to the extent of about 4 p.p.m. in the final solution, had interfered with the estimation of nitrite. On the other hand, silver nitrate showed much more inhibition by this method than by nitrite estimation with JR as the carrier.

The nitrophenols, none of which were reduced by the enzyme, were strongly inhibitory. Lascelles (1946), however, had found that, measuring the rate of hydrogen uptake in the presence of nitrate and intact *E. coli* cells, nitrate reduction was not inhibited by nitrophenols.

Finally, it may be said that molar urethan causes slight inhibition. This has been confirmed with the oxidation time technique, when an inhibition of about 20 per cent. was found. However, this large concentration of urethan precipitated the enzyme (at least in the state in which it was used), so that in all probability urethan itself has only a very slight effect on the enzyme. This is not in agreement with Yamagata (1938a).

VI. OBSERVATIONS ON NITRITE REDUCTASE

Frequently, throughout this work, preparations were tested for nitrite reductase. The results obtained, however, were made doubtful by the experiments of Aubel, Schwartzenkopf, and Glaser (1937), who suggested that nitrite could re-oxidize leuco-dyes chemically.

TABLE 9
EFFECT OF DIFFERENT SUBSTRATES ON THE OXIDATION TIME*

Substrate	Oxidation Time with Enzyme Preparation (min. & sec.)			
	ES Ia	ES Ib	ES II	ES III
Nitrate	1-40	5-50	14-20	10-00
Nitrite	0-40	1-00	>60	>60
Hydroxylamine	24-30	34-55	>60	>60

* Each tube contained enzyme solution (0.1 ml. of ES Ia and b, 1.0 ml. of ES II and ES III; 0.5 ml. 0.1M substrate; 0.5 ml. 0.1M $\text{Na}_2\text{S}_2\text{O}_4$; 1.0 ml. 0.0004M JR; phosphate buffer (pH 7.6) to 4.0 ml.; and a layer of paraffin oil.

On the other hand it was shown repeatedly that, under the conditions used here, tubes containing buffer, nitrite, sodium hydrosulphite, and ORI, but no enzyme, did *not* re-oxidize the dyestuff. Hence Table 9 provides some evidence that under certain conditions nitrite reductase can be very active and is an entity different from nitrate reductase. The experiment was carried out with enzyme preparations which had just been obtained from crushed bacteria. Fractions ES Ia, ES Ib, ES II, and ES III have been described previously (Joklik 1950): they refer to ammonium sulphate fractions of the extract of crushed bacteria. E(nzyme) S(olutions) I were those before ammonium sulphate treatment, and ES III were suspensions in phosphate buffer (pH 7.6) of the precipitate obtained by between one-third and one-half saturation with ammonium sulphate; ES II fractions were intermediate. Suspensions of this type were by no means pure preparations of nitrate reductase, but they were convenient to prepare and other enzymes did not interfere.

It appears from Table 9 that, since the amounts of sodium hydrosulphite and substrate were the same in all tubes, an explanation based on *simple* chemical reduction of the substrate, is not adequate. However, in view of the extraordinarily short oxidation times in the presence of nitrite, it seems that the factors involved in its reduction are unknown. Experiments using photochemically reduced MB as hydrogen donor provided evidence of the presence of a weak nitrite-reducing enzyme in certain preparations. The best method of studying the enzyme at the present time would be with a purified preparation of hydrogenase, measuring the hydrogen uptake. Nevertheless, even then studies of the enzyme, such as pH optima, effect of inhibitors as well as the possible need for a carrier between the two enzymes, would be greatly complicated.

VII. RECONSTRUCTION OF THE HYDROGENASE-NITRATE REDUCTASE SYSTEM

With the separation of the two enzymes, it is now possible to reconstruct the system hydrogenase-nitrate reductase.

A number of workers have noticed dilution effects in studying reactions involving hydrogenase and nitrate reductase, but the nature of the carriers involved is entirely unknown. Hoberman and Rittenberg (1943) suggested that an *enzyme system*, and not only a carrier, is involved in the reduction of MB by hydrogenase. So far as nitrate reductase is concerned, the position is still more uncertain. Yamagata (1938*b*) states that reduced coenzyme I is not a donor and this fact has been confirmed here, while Green, Stickland, and Tarr (1934) found that except for oxidation-reduction indicators, pyocyanine was the only substance capable of transferring hydrogen atoms from dehydrogenases to nitrate reductase. Accordingly a short series of experiments was carried out using both enzymes in dialysed preparations.

Before carrying out experiments with the two purified enzymes, a dialysed fraction of ES I was tested for the effect of certain chemical reagents, from which, as Hoberman and Rittenberg suggest, "the hydrogen ion and electron pair, necessary for the reduction of MB" could be contributed. The dialysis of the ES I (which contained both hydrogenase and nitrate reductase) was done very carefully, first against distilled water containing a few drops of thioglycollic acid, then against running water for a short time, and finally for a few short periods against glass-distilled water; this was necessary to prevent excessive denaturation of hydrogenase. Table 10 shows the results.

Table 10 shows that none of these reagents except boiled bacteria had any effect on the reaction; the carrier responsible was thus not replaceable by any of the above reagents. The relatively large activity left after dialysis showed that this process was not the one which removed so much of the carriers that the purified preparations of the two enzymes, as is discussed below, only reacted together at a very low rate. A boiled bacterial suspension, however, gave a significant result, showing that some carrier involved in the transport of hydrogen was heat-stable and present in deficient amounts in the fraction ES I.

Experiments were then carried out with the two purified enzyme preparations. The hydrogenase used was of the type ES III (Joklik 1950), i.e. heat-treated, dialysed, and precipitated with ammonium sulphate; the nitrate reductase used was in the purest form available (after acetone precipitation).

TABLE 10
EFFECT OF VARIOUS CARRIERS ON THE HYDROGENASE-NITRATE REDUCTASE
REACTION IN DIALYSED SOLUTIONS CONTAINING BOTH ENZYMES*

Reagent Added	Hydrogen Uptake
None	84
0.3 ml. coenzyme I solution (15 mg./ml.)	86
0.1 ml. Nile blue (0.2%)	79
0.3 ml. coenzyme I solution + 0.1 ml. Nile blue	94
0.1 ml. fumarate (0.1M)	100
0.1 ml. fumarate + 0.3 ml. coenzyme I solution	90
0.3 ml. boiled suspension of bacteria	139

* Warburg manometers were used. Each cup contained the following reagents: dialysed enzyme preparation, 1.0 ml.; 0.1 ml. nitrate (0.1 M); 0.4 ml. MB (2%); where indicated, the reagents mentioned; and phosphate buffer (pH 7.6) to 3.0 ml. Incubation time was half an hour, and the readings are in $\mu\text{l.}/30 \text{ min.}$

Table 11 shows the results obtained. They suggest that flavoprotein is involved somewhere in the transport of hydrogen from the gaseous phase to nitrate; but it would be too early to assign to it a definite role either as carrier or as a prosthetic group of the enzyme.

TABLE 11
REACTION BETWEEN HYDROGENASE AND NITRATE REDUCTASE IN
PURIFIED PREPARATIONS*

Nitrate reductase	+	+	+	+	+
Hydrogenase	+	+	+	+	+
Reagent	None	BB	FP	FP Co I	NB Co I
Activity	13	33	38	43	35

* Warburg manometers were used. Each cup contained 0.5 ml. of nitrate reductase and hydrogenase solutions; 0.4 ml. MB (2%); where indicated the following: 0.3 ml. boiled suspension of bacteria (BB), 0.1 ml. flavoprotein solution (FP), 0.3 ml. coenzyme I solution (15 mg./ml.) (Co I), and Nile blue (0.3%) (NB); and phosphate buffer (pH 7.6) to 3.0 ml.

It is remarkable how slow the hydrogen uptake was under these conditions since the particular hydrogenase preparation used took up 250 $\mu\text{l.}$ in 30 minutes in the presence of MB, and the activity of the nitrate reductase was such that with 0.2 ml. of 0.1M sodium hydrosulphite the oxidation time was only 15 minutes; if these systems had reacted at something like the optimum speed for each, the hydrogen uptake would have been over ten times the value found. However, even in the presence of whole bacteria, the uptake of

gas is much greater in the presence of MB than in the presence of nitrate, so that even under conditions where the structural relationships between the two enzymes are preserved, the absence of a sufficient amount of carrier limits the rate of the reaction. Regarded in that way, activation with boiled suspensions of bacteria is not surprising.

VIII. DISCUSSION

The evidence presented here does not make it possible to speculate on the nature of nitrate reductase. The only observation which could strengthen the evidence for the presence of a prosthetic group in the enzyme would be that by precipitating the enzyme into acidified acetone relatively little activity is lost until a molarity of hydrochloric acid of 10^{-3} is reached (Section V (d)); in the step from there to 10^{-2} M, however, all activity disappears. This might indicate the splitting off of a prosthetic group rather than sudden enzymic denaturation. The claim of Lemoigne, Desveaux, and Gavard (1942), however, that nitrate reductase consists of two parts, one of which is in the cell-free culture fluid, could not be confirmed.

When the question of the nature of the prosthetic group is considered, two alternatives are open. On the one hand, the strong inhibition with cyanide and azide suggests that an iron porphyrin prosthetic group might be implicated; the enzyme is not, however, affected by carbon monoxide, nor by reagents for free iron. On the other hand, there is also evidence for a flavine prosthetic group, as indicated by the fact that flavoprotein serves as a hydrogen carrier between hydrogenase and nitrate reductase.

It is also of particular significance that the relation between nitrogen metabolism and molybdenum, which has been known to exist for some time in *Azotobacter* (Bortels 1930; Burk and Horner 1935), *Rhizobium* (Jensen and Betty 1943), and *Clostridium* (Jensen and Spencer 1947), and in particular that between nitrate metabolism and molybdenum (Bortels 1936; Jensen and Spencer 1947) has been recently observed in *E. coli* (Pinsent 1949).

The only definite statement which can be made about the structure of the enzyme at the present time, is that free -SH groups do not seem to be necessary for enzymic activity, at least not to the extent of enabling the enzyme to be classed with the -SH enzymes. It is also interesting that nitrophenols inhibit the reduction of nitrate; this was rather unexpected, since they do not markedly affect the reduction of nitrate in intact cells (Lascelles and Still 1946). Toluene likewise did not cause any inhibition; Lascelles and Still (1946) had found slight inhibition, while Yamagata (1938a) claimed that it inhibited appreciably.

As far as carriers between hydrogenase and nitrate reductase are concerned, only dyes and flavoprotein seem to be active. An unidentified carrier occurs in boiled bacteria, which also increases the rate of interaction between nitrate reductase and other enzymes.

The physiological significance of nitrate reductase is fairly clear, as was shown by Quastel, Stephenson, and Whetham (1925); it may be assumed

that the reaction between hydrogen and nitrate rarely, if ever, proceeds under physiological conditions. The function of the enzyme might be summed up by the statement that since its E° is quite high (about 0.4 volts), which places it above most dehydrogenases in the scale of oxidation-reduction potentials, it can act as a terminal oxidizing agent under anaerobic conditions.

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THE BACTERICIDAL PROPERTIES OF CERTAIN CATIONIC DETERGENTS

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Summary

Using the F.D.A. method of testing germicides, the bactericidal properties of two cationic detergents, "Cetavlon" and "Fixanol C," were tested against ten organisms at seven pH levels between 5.2 and 8.2. The results for the two detergents were similar. Gram-positive organisms were more susceptible than Gram-negative. There was considerable variation in the relationship between susceptibility and pH. *Staph. aureus*, *Staph. albus*, *Strep. faecalis*, and *Proteus vulgaris* were all most susceptible under slightly alkaline conditions; whereas *Ps. fluorescens*, *Ps. pyocyanea*, *Achromobacter liquefaciens*, and *Bacterium coli* were most susceptible under slightly acid conditions. The susceptibility of *Corynebacterium equi* was unaffected by pH within the range studied.

One strain each of *Staph. aureus* and of *Ps. fluorescens* were studied in more detail. Plate count disinfection studies confirmed the greater rate of destruction of *Staph. aureus* at pH 8.1, and of *Ps. fluorescens* at pH 5.3. Neither of two anionic detergents reversed the bactericidal action of "Cetavlon." For both types of cells the adsorption of detergent was greatest at pH 8.2. Likewise the inhibition of oxygen uptake showed no marked relationship with mortality. Treatment with detergent caused an increased loss of phosphorus-bearing compounds from both types of cells, but the data are insufficient to show whether susceptibility to the detergent was related to this leakage from the cells.

I. INTRODUCTION

The bactericidal properties of synthetic detergents have been the subject of numerous investigations since Domagk (1935) reported that the cationic detergent "Zephiran" possessed germicidal activity. Factors of molecular structure, such as the length of the alkyl chain and the nature of the anion central atom, have been found to influence the bactericidal activity, and this work has been reviewed by Glassman (1948). The influence of pH on the germicidal efficiency of both cationic and anionic detergents has been reported in many investigations (Gershenfeld and Perlstein 1941*a*, 1941*b*; Gershenfeld and Milanick 1941; Baker, Harrison, and Miller 1941*a*, 1941*b*; Hoogerheide 1945; Quisno and Foter 1946; Eisman and Mayer 1947).

It has been generally accepted from these investigations that, as one departs from a neutral pH, the cationic detergents become more effective with increasing alkalinity, whereas the reverse is true for the anionic detergents.

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However, Quisno and Foter (1946) reported that cetylpyridinium chloride is germicidal in high dilutions under acid as well as alkaline conditions and maintains an even level of bactericidal activity over the pH range 2 to 10. Apart from this finding with cetylpyridinium chloride, there would appear to be a similarity between the cationic and anionic detergents on the one hand, and the basic and acid dyes on the other in their pH-bactericidal relationships. Stearn and Stearn (1924) showed that an increase of pH favoured the disinfecting power of basic dyes, whilst a converse relationship was found with acid dyes.

Baker, Harrison, and Miller (1941a) examined the effect of cationic and anionic detergents on the metabolism of Gram-positive and Gram-negative bacteria and found that respiratory and glycolytic activity were markedly reduced. These authors found that inhibition of oxygen uptake was greatest at pH 8.0. In a later work, Baker, Harrison, and Miller (1941b) concluded that inhibition of metabolism and destruction of the bacteria were roughly parallel. As Hotchkiss (1946) pointed out, the regularity of this relationship was impaired by frequent observations of the lack of bactericidal activity under the same conditions which markedly inhibited respiration.

The observations that the Gram-negative organism *Pseudomonas fluorescens* was more susceptible to the bactericidal activity of a cationic detergent under acid than under alkaline conditions suggested the possibility that other organisms may also differ in their pH-bactericidal relationships. This led to the present study in which adsorption of detergent, inhibition of respiration, and loss of cellular phosphorus have each been studied in relation to the antibacterial activity at various levels of pH.

II. EXPERIMENTAL METHODS

The two cationic detergents used in these investigations were "Cetavlon" (cetyltrimethylammonium bromide) and "Fixanol C" (cetylpyridinium bromide) manufactured by Imperial Chemical Industries. "Cetavlon" was in the form of a white crystalline powder, and "Fixanol C" was a brown paste. Determination of the content of cation-active agent in both compounds was done by titration based on the assay described by Auerbach (1943). Both "Cetavlon" and "Fixanol C" contained approx. 75 per cent. cation-active agent.

The following organisms were used in the initial studies in the effect of pH on the bactericidal properties of "Cetavlon" and "Fixanol C" determined by the United States Food and Drug Administration method.

Gram-positive organisms: *Staphylococcus aureus*, *Staphylococcus albus*, *Streptococcus faecalis*, *Corynebacterium equi*; Gram-negative organisms: *Pseudomonas fluorescens* H 137, *Pseudomonas fluorescens* 542, *Pseudomonas pyocyanea*, *Bacterium coli*, *Proteus vulgaris*.

Throughout this study of the bactericidal properties of cationic detergents M/15 disodium hydrogen phosphate-potassium dihydrogen phosphate buffers have been used, and will be referred to subsequently as 'phosphate buffer.'

(a) *Food and Drug Administration Method*

The Food and Drug Administration method, described by Ruehle and Brewer (1931), was used in determining the minimum concentration of detergent which produced sterilization of 22-26 hr. cultures in 10 min., but not in 5 min.

The following technique was used: 5.0 ml. of each concentration of detergent was sterilized in phosphate buffer adjusted to seven levels of pH ranging from pH 5.2 to 8.2. To each tube was added 0.5 ml. of a 22-26 hr. culture of the test organisms in F.D.A. broth (containing 0.3 per cent. "Difco" beef extract and 0.5 per cent. "Bacto-Peptone"). At intervals of 5, 10, and 15 min. one loopful (from a standard loop 4 mm. internal diameter) of approx. 0.01 ml. volume was transferred to fresh broth. After incubation for a period of 48-72 hr. the tubes were examined for turbidity and the minimum concentrations showing sterilization in 10 min. and not in 5 min. were recorded. All experiments were performed at 30°C.

For some organisms plate counts of the 22-26 hr. F.D.A. broth cultures were made.

(b) *Rate of Disinfection*

A study of the rate of disinfection of the two organisms, *Staph. aureus* and *Ps. fluorescens* 542, by "Cetavlon" was made at four levels of pH. For these studies the organisms were grown on fresh meat agar (medium prepared from minced ox heart infusion 500 g., peptone 15 g., sodium chloride 5 g., agar 10 g. (from Davis Gelatine (N.Z.) Ltd., Christchurch, N.Z.), made up to 1 litre, the pH adjusted to 7.6-7.8), cultures being incubated at 30°C. for 18 hr. Cells were harvested by washing from the surface of the agar with saline and centrifuging. Two washings in saline followed and the cells were finally resuspended in saline.

Phosphate buffer (M/15) adjusted to levels of pH 5.3, 6.2, 7.2, and 8.2, containing "Cetavlon" to give a final concentration of 100 µg./ml., was sterilized prior to inoculation with the bacterial suspension of which 1.0 ml. was added to 9.0 ml. of the "Cetavlon" solutions. Samples of 1.0 ml. were withdrawn at various intervals and transferred to 99 ml. of saline. Dilutions for the determination of the numbers of survivors were plated out in brain heart infusion agar and counted after 48-72 hr. at 30°C. All experiments on the rates of disinfection were performed at 30°C.

(c) *Effect of Anionic Detergents on Treated Cells*

Several experiments with *Ps. fluorescens* 542 were carried out to test the effect of the two anionic compounds, "Aerosol OT" and sodium cetyl sulphate, on the numbers of organisms surviving 15 min. exposure to "Cetavlon" at 30°C. Suspensions of *Ps. fluorescens* 542 were prepared as for the disinfection rate studies. In one experiment, samples of bacteria were pipetted into 99 ml. saline containing 2 µg./ml. "Aerosol OT," after having been exposed to solutions containing 100 and 200 µg. "Cetavlon"/ml. Dilutions were plated on

brain heart infusion agar. In another experiment, dilutions of bacteria exposed to "Cetavlon" (100 $\mu\text{g./ml.}$) for 15 min. at 30°C. were plated out in brain heart infusion agar containing 10 and 100 $\mu\text{g./ml.}$ of "Aerosol OT" and sodium cetyl sulphate. For both experiments "Cetavlon" was in phosphate buffer at pH 7.

(d) Adsorption

Determinations of the amount of "Cetavlon" adsorbed by the two organisms, *Staph. aureus* and *Ps. fluorescens* 542, were made using the colorimetric assay of quaternary ammonium salts described by Auerbach (1944).

Organisms were grown on fresh meat agar and harvested after 18 hr. incubation at 30°C., washed twice, and resuspended in saline, as for the disinfection rate studies. 1.0 ml. of bacterial suspension was added to 9.0 ml. of "Cetavlon" in phosphate buffer, to give final concentrations of 100, 200, 400, and 800 $\mu\text{g.}$ "Cetavlon"/ml. After 15 min. exposure to "Cetavlon" at 30°C., samples were taken for estimations of the numbers of survivors. The bacteria-"Cetavlon" mixture was then transferred to centrifuge tubes, the cells removed, and estimations of the residual quaternary ammonium salt in the supernatant were made. These experiments were performed at pH levels of 5.2, 6.2, 7.2, and 8.2.

This method of measuring the adsorption of quaternary ammonium compounds by suspensions of bacteria is apparently similar to the method used by Valko and Dibblee (unpublished data) referred to by Valko (1946).

(e) Inhibition of Respiration

Inhibition of oxygen uptake by suspensions of *Staph. aureus* and *Ps. fluorescens* 542 was measured manometrically. Organisms were grown on fresh meat agar, harvested after 18 hr. incubation at 30°C., washed twice, and resuspended in saline.

The manometric technique was essentially the same as that used by Baker, Harrison, and Miller (1941a) in their studies on the action of synthetic detergents on bacterial metabolism. Phosphate buffer adjusted to pH levels of 5.2, 6.2, 7.2, and 8.2 was used; 1.7 ml. of buffer being added to each Warburg flask. 0.1 ml. of 0.6M glucose solution (making final concentration of glucose 0.02M), 1.0 ml. bacterial suspension, 0.2 ml. "Cetavlon" in phosphate buffer (in side-arm), and 0.3 ml. of 20 per cent. (w/v) KOH solution (centre well) were added to each flask. Two flasks for each treatment were prepared. Oxygen uptake at 30°C. was followed over a period of one hour, readings being recorded at 10 min. intervals.

At the conclusion of the manometric observations, suspensions from the two flasks were pooled and samples were taken for the estimation of the number of surviving bacteria.

(f) Loss of Phosphorus-containing Compounds from Treated Cells

The bacteria were grown on fresh meat agar to which was added radioactive phosphorus (P^{32}) in the form of H_3PO_4 . Cells were harvested after 18

hr. incubation at 30 C., washed twice, and resuspended in saline as for previous studies.

The amount of P^{32} taken up by the cells was determined by drying (at 100°C.) suitable aliquots of the suspension on discs and recording Geiger-Mueller counts. The loss of P^{32} from bacterial cells exposed to a "Cetavlon" concentration of 100 μ g./ml. at 30 C. for 15 min. was determined by recording Geiger-Mueller counts on aliquots of filtrate obtained by removing the cells with sintered glass filters. These observations were made with phosphate buffer at pH levels of 5.2 and 8.2 in the presence and absence of "Cetavlon." Five counts per aliquot were made with the Geiger-Mueller counter, the aliquots being adjusted, where possible, to give counts of the order of 300/min.

The numbers of survivors were again estimated at the end of the 15 min. exposure period, the samples being withdrawn just prior to filtration.

III. RESULTS

(a) Food and Drug Administration Method

The results for ten organisms are shown in Figures 1 and 2 for "Cetavlon" and "Fixanol C" respectively. Each curve is drawn through seven points, each of which represents the arithmetic mean of not less than four determinations of the concentration of detergent which resulted in sterile sub-cultures after ten, but not after five, minutes exposure at the pH specified. Although the points often fall on a reasonably smooth curve, irregularities characteristic of such "end-point" determinations were recorded frequently. In this respect the experience is similar to that reported by Quisno and Foter (1946) and Klarmann and Wright (1946).

TABLE 1
PLATE COUNTS OF ORGANISMS GROWN IN F.D.A. BROTH

Organism	No. of Estimates	Mean Population/ml. of Culture
Gram-positive:		
<i>Staph. aureus</i>	11	4.3×10^8
<i>Strep. faecalis</i>	9	2.4×10^8
<i>Corynebacterium equi</i>	10	2.7×10^6
Gram-negative:		
<i>Ps. fluorescens</i> 542	11	5.4×10^8
<i>Ps. pyocyanea</i>	10	1.0×10^9
<i>Proteus vulgaris</i>	9	6.8×10^8
<i>Achromobacter liquefaciens</i>	13	6.9×10^8

Approximate estimates of the numbers of each organism added to each tube of disinfectant were obtained by plate counts of the cultures used as inoculum. The average values for the plate counts are shown in Table 1. As

0.5 ml. of broth was added to 5 ml. of detergent solution, and the sample for the test of sterilization was approximately 0.01 ml., the initial number of viable cells per loop sample is approximately 1/1000 of the plate counts listed. It is apparent, therefore, that under F.D.A. test conditions the initial number of viable cells will vary from one organism to another, and that differences as great as 100-fold may be recorded.

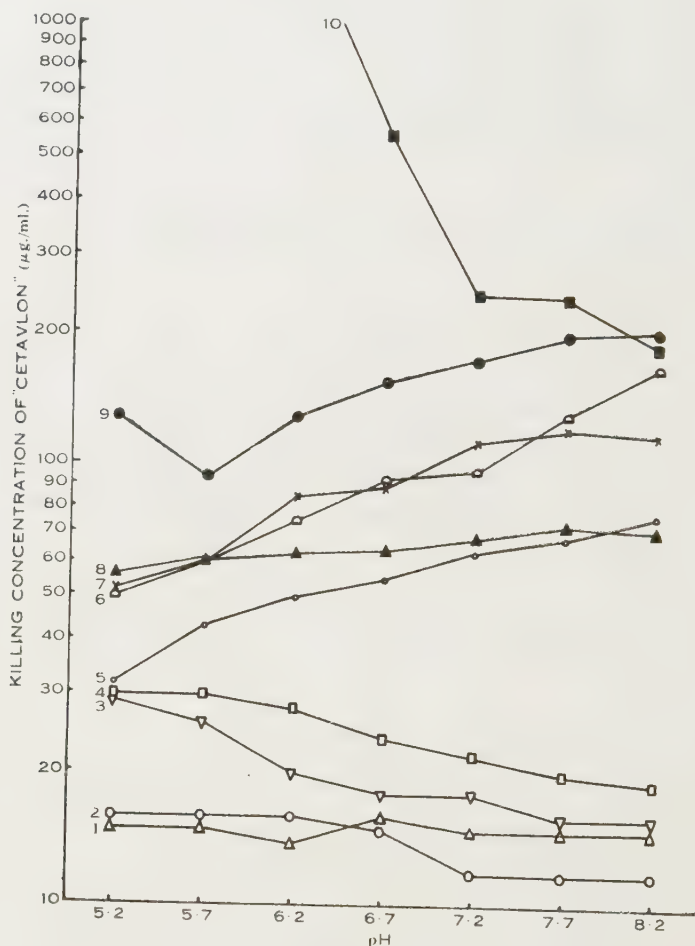


Fig. 1.—F.D.A. killing concentrations of "Cetavlon" for ten bacteria. 1. *Corynebacterium equi*; 2. *Streptococcus faecalis*; 3. *Staphylococcus albus*; 4. *Staphylococcus aureus*; 5. *Pseudomonas fluorescens* H137; 6. *Pseudomonas fluorescens* 542; 7. *Achromobacter liquefaciens*; 8. *Bacterium coli*; 9. *Pseudomonas pyocyanea*; 10. *Proteus vulgaris*.

Figures 1 and 2 indicate a marked similarity between the two compounds tested, and some substantial differences between the organisms. For both compounds the Gram-positive organisms are generally more susceptible than Gram-negative organisms, a finding which confirms earlier reports. The

susceptibilities of the various organisms also show considerable differences in relation to pH. *Staph. aureus*, *Staph. albus*, *Strep. faecalis*, and *Proteus vulgaris* are more susceptible under slightly alkaline than under slightly acid conditions, such results being in accord with the findings of Gershenfeld and Milanick (1941), and Gershenfeld and Perlstein (1941a, 1941b) for *Staph. aureus*. For *Ps. fluorescens*, *Ps. pyocyanea*, *A. liquefaciens*, and *Bact. coli* a converse relationship applies, and the organisms are more susceptible in a

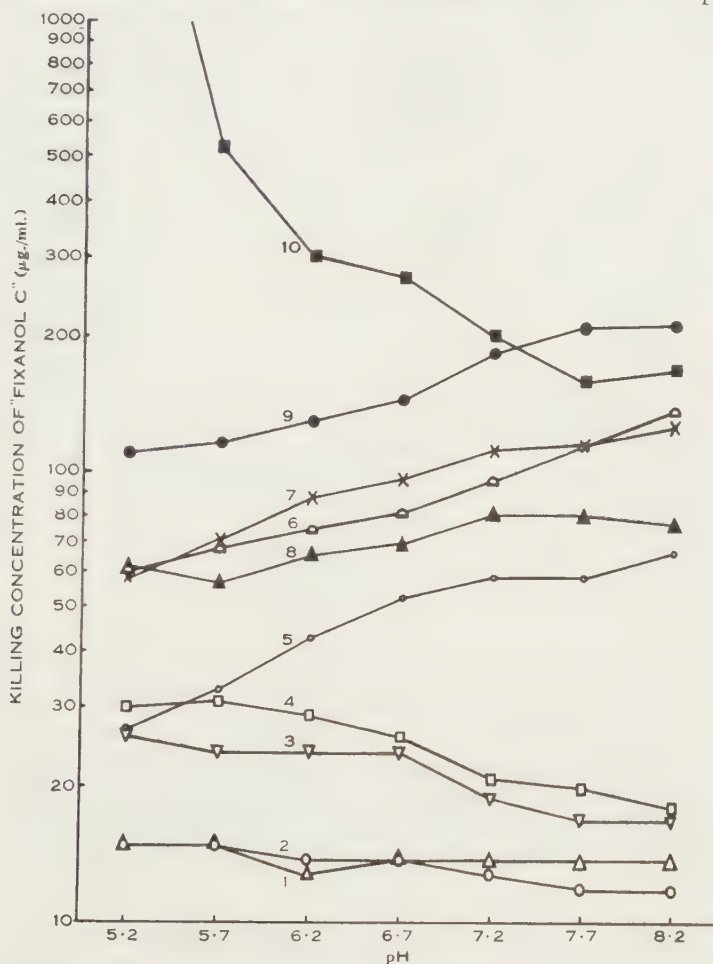


Fig. 2.—F.D.A. killing concentrations of "Fixanol C" for ten bacteria. Symbols as for Figure 1.

slightly acid environment. Although Quisno and Foter (1946) report that the bactericidal activity of cetylpyridinium chloride against *Staph. aureus* and *E. typhosa* was virtually unaffected by pH in the range 2 to 10, there appear to be no reports of increased bactericidal effects under slightly acid conditions. No significant change in the susceptibility of *Corynebacterium equi* is apparent over the pH range studied.

(b) Rate of Disinfection

The principal aim of studying the rate of disinfection was to determine whether the pH-susceptibility relationships indicated by the F.D.A. method were revealed also by plate-count estimates of survivors after various times of exposure. The experiments were carried out, therefore, with two organisms (*Staph. aureus* and *Ps. fluorescens* 542) showing, by the F.D.A. technique, different susceptibility-pH relationships. Estimates of survivors were made when the initial populations were between 10^9 and 10^{10} cells/ml., and also with smaller initial populations between 10^7 and 10^8 /ml. Figures 3 and 4 show the results for *Staph. aureus* and *Ps. fluorescens* 542 respectively, each point representing the average of four determinations for the higher initial population and two for the lower.

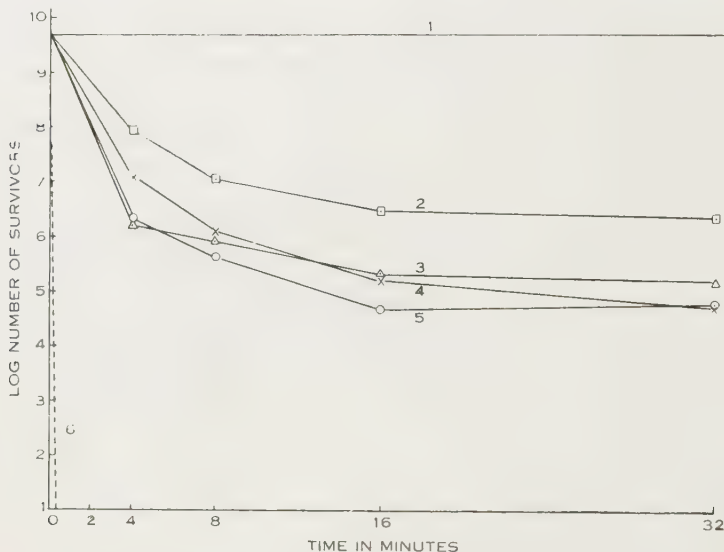


Fig. 3.—Disinfection of *Staph. aureus* by 100 μ g./ml. of "Cetavlon."
1. Controls (no "Cetavlon") at four pH levels; 2. "Cetavlon" at pH 5.3; 3. "Cetavlon" at pH 7.2; 4. "Cetavlon" at pH 6.2; 5. "Cetavlon" at pH 8.1; 6. Initial number of cells $10^{9.7}$ /ml. "Cetavlon" at all four pH levels.

Figure 3 shows that for an initial population of $10^{9.7}$ /ml. the rate of decrease of the survivors is least at pH 5.3, with little, if any, difference between the rates at the three higher pH levels. When the logarithms of the numbers of survivors were plotted against the cube root of the exposure time, significant linear regressions were obtained, the slope of the curve being significantly less at pH 5.3 than at pH 6.2, 7.2, and 8.1. It will be noted that the rate of death gradually falls, and the population of survivors becomes virtually constant after 16 minutes. This decline in the rate of death is no doubt due to the fact that this concentration of cells is sufficient to adsorb almost all the detergent from solution during the first few minutes (see Sub-section III(d))

below). When the initial number of cells was reduced to approximately $10^{7.7}$ /ml. the number of survivors was less than 100/ml. after 30 seconds exposure at all four pH levels. This greater rate of death is undoubtedly due to the fact that the actual concentration of detergent would be almost unaffected by adsorption.

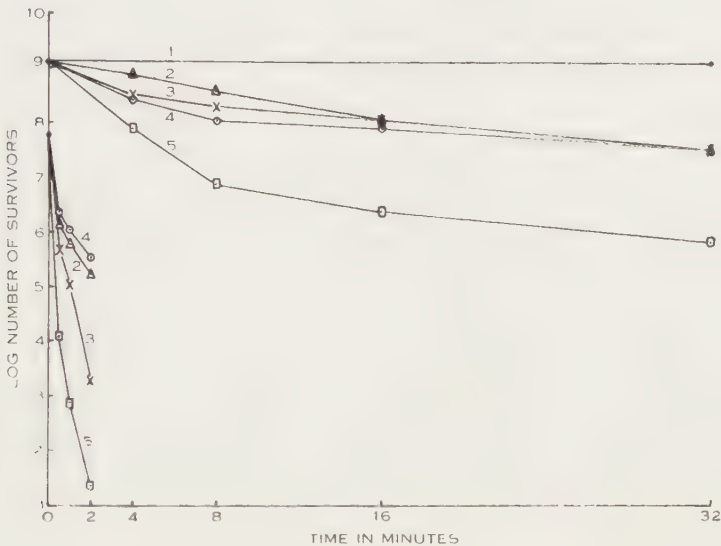


Fig. 4.—Disinfection of *Ps. fluorescens* 542 by 100 µg./ml. of "Cetavlon." 1. Controls (no "Cetavlon") at four pH levels; 2. pH 7.2; 3. pH 6.2; 4. pH 8.0; 5. pH 5.3.

In Figure 4 the data for *Ps. fluorescens* 542 show that the greatest rate of death occurs at pH 5.3 for initial populations of 10^9 and $10^{7.7}$ /ml. For the higher initial population adsorption of most of the detergent again resulted in rates of death which decrease substantially with increasing time of exposure. Linear regressions were obtained when the logarithms of the numbers of survivors were plotted against the square root of the exposure time, the slope of the curve being significantly greater at pH 5.3 than at the three higher pH levels. When the initial number of cells was reduced to $10^{7.7}$ /ml. the rate of death was substantially increased at all pH levels, that at pH 5.3 being greater than at pH 6.2, and this in turn is greater than the rates at pH 7.2 and 8.0.

These results, therefore, provided independent confirmation of the two types of pH-susceptibility relationships indicated by the F.D.A. method.

(c) Effect of Anionic Detergents on Treated Cells

Several experiments with *Ps. fluorescens* 542 were performed to investigate the possible reversal of bactericidal action by anionic detergents, as had been found for *Staph. aureus* by Valko and Dubois (1944). For cells exposed at

pH 7 to "Cetavlon" (100 $\mu\text{g./ml.}$), resulting in from 1 to 10 per cent. survivors after 15 min., dilution in a solution containing sufficient "Aerosol OT" to neutralize the "Cetavlon" was without effect on the number of survivors. Similarly, the incorporation of both "Aerosol OT" and sodium cetyl sulphate in brain heart agar (10 and 100 $\mu\text{g./ml.}$) had no detectable influence on the number of survivors. In a single experiment prior exposure to "Aerosol OT" afforded no protection to cells subsequently treated with "Cetavlon." Under these conditions, therefore, there is no indication that the anionic detergents will reverse the bactericidal action of "Cetavlon."

(d) Adsorption of Detergent by Bacteria

Measurement of adsorption of "Cetavlon" by *Ps. fluorescens* 542 after 15, 30, and 45 min. showed that adsorption was virtually complete in 15 min. Estimates of the amounts adsorbed within shorter periods were not feasible with the technique used.

The results for the adsorption of "Cetavlon" by *Staph. aureus* and *Ps. fluorescens* 542, using total cell populations of $3\text{--}5 \times 10^9/\text{ml.}$ at four levels of pH are shown in Figures 5 and 6. From inspection of these adsorption isotherms it is evident that adsorption is proportional to the concentration and independent of pH up to approx. 200 $\mu\text{g./ml.}$ initial concentration. As the

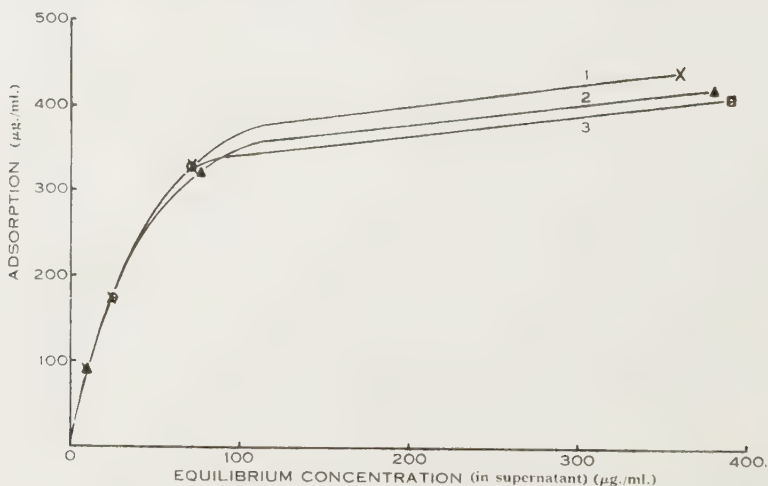


Fig. 5.—Adsorption of "Cetavlon" by approximately 5×10^9 cells of *Staph. aureus* at four levels of pH. 1. pH 8.2; 2. pH 7.2; 3. pH 6.2 and 5.2.

concentration is increased, maximum adsorption is achieved, the level of which is dependent on the pH. For both organisms the greatest capacity for detergent is manifest at pH 8.2. At this pH, *Staph. aureus* has adsorbed 1.3×10^8 molecules/cell and *Ps. fluorescens* 542, 2.2×10^8 molecules/cell. These amounts are rather more than the approximations of detergent per bacterial cell corresponding to a monolayer. It is of interest to note that McMullen and

Alexander (1949) found the amount of "Aerosol OT" adsorbed by *Staph. aureus* at the highest concentration studied corresponded to about one mono-

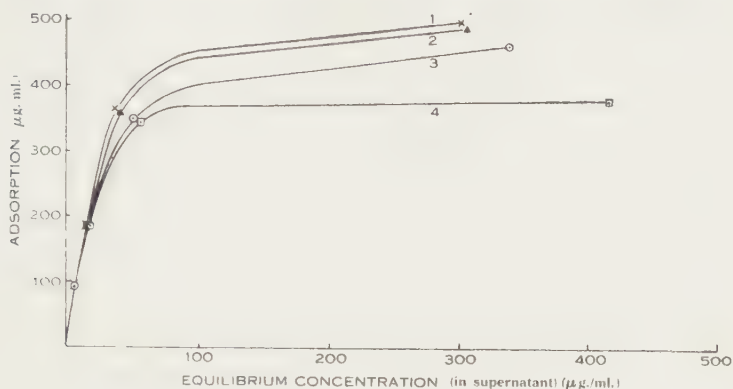


Fig. 6.—Adsorption of "Cetavlon" by approximately 3×10^9 cells of *Ps. fluorescens* 542 at four levels of pH. 1. pH 8.2; 2. pH 7.2; 3. pH 6.2; 4. pH 5.2.

layer. Parallel determinations were made on the same suspension of both adsorption and mortality after exposure for 15 min. at each of four concentrations of "Cetavlon" and four levels of pH. The average results for two experiments are shown in Table 2 for *Staph. aureus* and in Table 3 for *Ps. fluorescens* 542.

TABLE 2
INFLUENCE OF pH ON THE NUMBER OF SURVIVORS AND ADSORPTION OF "CETAVLON" BY *STAPH. AUREUS*

pH	Concentration of Added "Cetavlon" (μg./ml.)								
	0	100		200		400		800	
	Log p*	Log p	Adsorp- tion†	Log p	Adsorp- tion	Log p	Adsorp- tion	Log p	Adsorp- tion
5.2	9.7	8.3	90	6.5	176	<2.0	328	<2.0	410
6.2	9.6	6.4	89	5.8	176	"	328	"	410
7.2	9.5	5.2	90	3.7	174	"	323	"	420
8.2	9.6	4.7	92	3.7	175	"	328	"	440

* = log. of plate count/ml., mean of two experiments.

† = amount adsorbed in μg./ml., mean of two experiments.

Although the amount of detergent adsorbed by the cells of both organisms is virtually independent of pH at the lower concentrations of 100 and 200 μg./ml., the numbers of survivors are dependent on pH. Table 2 shows that the disinfection of *Staph. aureus* by both 100 and 200 μg./ml. has been most effective at pH 8.2 and least effective at pH 5.2. At the higher concentrations of detergent less than 100 survivors/ml. were recorded at all pH levels. On the other hand, Table 3 shows that the greatest destruction of *Ps. fluorescens* 542 occurred at pH 5.2 at each of the four concentrations tested.

It is evident, then, that the bactericidal activity of "Cetavlon" at various levels of pH is not correlated with the capacity of the cells to adsorb the detergent from solution.

TABLE 3
INFLUENCE OF pH ON THE NUMBER OF SURVIVORS AND ADSORPTION
OF "CETAVLON" BY *PS. FLUORESCENS* 542

pH	Concentration of Added "Cetavlon" (µg./ml.)								
	0	100		200		400		800	
	Log p*	Log p	Adsorp- tion†	Log p	Adsorp- tion	Log p	Adsorp- tion	Log p	Adsorp- tion
5.2	9.3	7.2	94	5.1	182	4.3	345	<2.0	385
6.2	9.4	7.3	94	5.9	183	5.1	350	4.1	463
7.2	9.4	8.3	94	7.8	185	5.5	360	3.0	495
8.2	9.2	8.7	95	7.5	187	5.2	364	2.5	500

* = log. of plate count/ml., mean of two experiments.

† = amount adsorbed in µg./ml., mean of four experiments.

(e) Effect of Detergent on Respiration

The relationship between inhibition of oxygen uptake (using glucose as substrate) and disinfection was studied at pH levels of 5.2, 6.2, 7.2, and 8.2. Figures 7 and 8 show the control and inhibited rates of oxygen uptake for *Staph. aureus* and *Ps. fluorescens* 542 respectively.

At the end of the manometric observations, samples were taken for the estimation of the numbers of survivors. The average results for two experiments are shown in Table 4.

TABLE 4
THE NUMBERS OF SURVIVORS AFTER 60 MINUTES EXPOSURE TO "CETAVLON" IN
MANOMETRIC EXPERIMENTS

Organism	Concentration of "Cetavlon" (µg./ml.)	Log Number of Survivors			
		pH 5.2	pH 6.2	pH 7.2	pH 8.2
<i>Staph. aureus</i>	0	9.70	9.73	9.66	9.71
	17	9.64	9.69	9.72	9.70
	25°	9.72	9.64	9.67	9.62
	33	8.97	8.93	8.80	9.05
<i>Ps. fluorescens</i> 542	0	8.71	8.84	8.76	8.80
	17	8.48	8.67	8.72	8.78
	33	7.26	8.40	8.55	8.18

° Result for one experiment only; log no. survivors for control was 9.74.

It is apparent from Figure 7 that pH has had little effect on the control and inhibited rates of oxygen uptake by *Staph. aureus* and there is no marked relationship between inhibition of respiration and destruction of the bacteria. These results with "Cetavlon" differ from those reported by Baker, Harrison,

and Miller (1941a) for "Zephiran" and "Emulsol 606." These authors found that for these two detergents inhibition of oxygen uptake by *Staph. aureus* was greatest at pH 8.0.

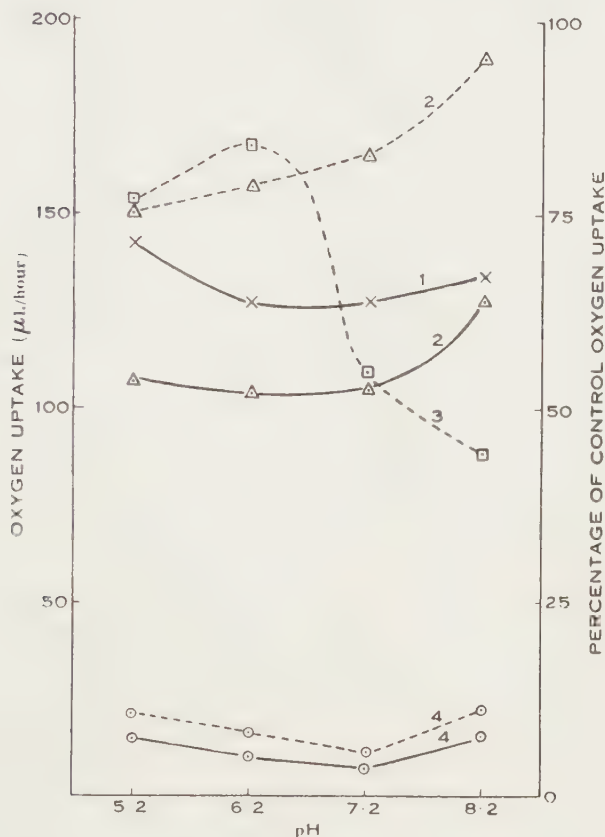


Fig. 7.—Effect of "Cetavlon" on oxygen uptake by *Staph. aureus*. Oxygen consumed in $\mu\text{l./hour}$: 1. No "Cetavlon"; 2. 17 $\mu\text{g./ml.}$ "Cetavlon"; 3. 25 $\mu\text{g./ml.}$ "Cetavlon"; 4. 33 $\mu\text{g./ml.}$ "Cetavlon." Oxygen consumed as percentage of control is shown by the broken lines.

Figure 8 shows that pH has a marked effect on the control rate of oxygen uptake of *Ps. fluorescens* 542. For this organism the greatest inhibition of respiration by "Cetavlon" occurred at pH 5.2 and this coincided with greatest bactericidal activity. However, at the three higher pH levels there is no obvious correlation between respiration inhibition and mortality.

(f) Loss of Phosphorus-containing Compounds from Cells

The loss of phosphorus-containing compounds from cells treated with "Cetavlon," and estimates of cells surviving exposure to the detergent are shown in Table 5. For both organisms treatment with "Cetavlon" has in-

creased the loss of phosphorus from the cells, although the amount released was only a small fraction of the original phosphorus content of the cells. The data are, however, insufficient to show whether the pH-susceptibility relationships are correlated with leakage of phosphorus from the treated cells.

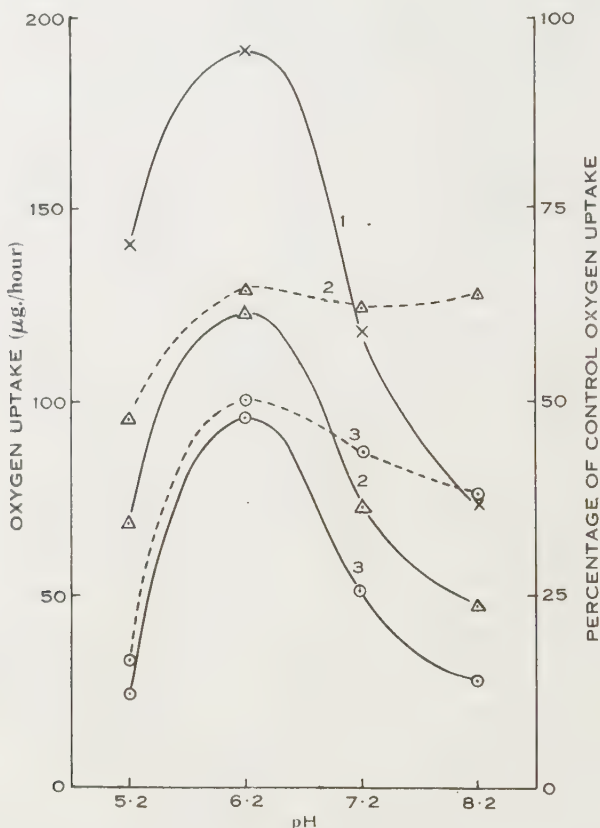


Fig. 8.—Effect of "Cetavlon" on oxygen uptake by *Ps. fluorescens* 542. 1. No "Cetavlon"; 2. 17 $\mu\text{g./ml.}$ "Cetavlon"; 3. 33 $\mu\text{g./ml.}$ "Cetavlon." Oxygen consumed as percentage of control is shown by the broken lines.

IV. DISCUSSION

Consideration of the results of Quisno and Foter (1946), and of those reported here shows that all bacteria do not show an enhanced susceptibility to cationic detergents when exposed under alkaline, rather than acid, conditions. Indeed, it appears that for some organisms the converse relationship exists. In the light of these facts it is difficult to propose a theory which would explain the mode of action of these surface active compounds.

The bacteriostatic activity of basic dyes increases with pH along with an increased adsorption of dye by the bacterial cell (Stearn and Stearn 1924). Valko and Dubois (1944) have suggested that the cationic detergents act in

a like manner, and it is true that the capacity of the bacterial cell to combine with "Cetavlon" increases as the pH is raised from 5 to 8. The bactericidal effects of "Cetavlon" are, however, marked at low concentrations where adsorption is apparently independent of pH, and, as shown in this paper, the anti-bacterial effects do change with pH, and in a direction which is dependent on the type of bacteria. It seems then that the anti-bacterial action of these detergents cannot be attributed simply to combination with oppositely charged groups on the bacterial surface.

TABLE 5
LOSS OF PHOSPHORUS COMPOUNDS AND NUMBERS OF SURVIVORS OF
"CETAVLON"-TREATED CELLS*

Organism	Expt. No.	pH 5.2 "Cetavlon" (μg./ml.)				pH 8.2 "Cetavlon" (μg./ml.)			
		0		100		0		100	
		P ³²	Log p†	P ³²	Log p	P ³²	Log p	P ³²	Log p
<i>Staph. aureus</i>	2	0.8	9.7	5.6	6.7	6.4	9.6	7.3	4.7
	3	0.8	9.5	9.3	6.1	6.8	9.4	14.5	4.1
<i>Ps. fluorescens</i> 542	1	5.7	9.0	17.2	6.4	2.2	9.1	9.3	8.0
	3	2.6	9.1	8.2	6.6	4.2	9.1	8.5	8.0

* P³² released from organisms expressed as a percentage of the P³² content of the cells.

† p = plate count/ml. after 15 minutes exposure at 30°C.

The adsorption of detergent as measured chemically, provides no evidence regarding the distribution of the compound between the various types of centres on the bacterial surface. The possibility exists then that combination with one particular type of centre may be of vital importance to the cell. It is unlikely that any such group of vital importance is directly concerned with respiratory activity as the inhibition of oxygen uptake at various pH levels has shown no clear relation with bactericidal effects. Hotchkiss (1946) has also interpreted the evidence of Baker, Harrison, and Miller (1941a) as being against the hypothesis that a key enzyme is especially sensitive to the detergent.

It has been shown that, in bactericidal concentrations, the detergents promote the leakage of cellular constituents, including nitrogen and phosphorus compounds (Hotchkiss 1944) and amino acids (Gale and Taylor 1947). Although these authors used Gram-positive organisms only, it is possible that Gram-negative organisms are affected in a similar way. Further investigation of the nature of the cytolytic damage suffered by both Gram-positive and Gram-negative cells would seem to be desirable, and may yet lead to an understanding of the varied pH-bactericidal relationships.

V. ACKNOWLEDGMENTS

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THE HISTOLOGY OF A VERY LARGE INSECT, *MACROPANESTHIA RHINOCERUS* SAUSS. (BLATTIDAE)

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Summary

The internal anatomy and histology of a large cockroach, *Macropanesthia rhinocerus* Sauss. (weight, approximately 20 g.), has been compared with that of a relatively small one, *Blattella germanica* (L.) (weight, approximately 0.06 g.). The observations have been considered in the light of Rensch's generalizations concerning histological changes in relation to body size and to the factors which may limit the size of insects. It is concluded that gravity, diffusion, and area for secretion and absorption are not factors limiting the size of *Macropanesthia*. The specializations observed in *Macropanesthia* are related to its burrowing habit and its food rather than to its large size.

I. INTRODUCTION

The factors which limit the size of insects have not been extensively studied. The difference in size between the smallest and largest insects is of the same order of magnitude as the difference in size between the smallest and largest mammals. Further, the smallest insects are smaller than the largest protozoa, and the largest insects are larger than the smallest mammals (Folsom 1922). Insects, therefore, provide suitable material for the study of the problems of the effect of size on organisms, and Rensch (1948) has referred to them extensively in suggesting a number of generalizations concerning the effects of size on histological structure.

Some of the problems involved in limiting the size of organisms have been discussed in a delightful essay "On being the Right Size" by J. B. S. Haldane (1927), who points out the paucity of data on the subject. He refers to some of the problems facing a large insect, notably respiration, locomotion, and temperature control; and the problems of tracheal respiration have also been considered in some detail by Krogh (1920).

A study of the factors which place an upper limit on insect size has more than theoretical interest, since it may indicate points in their physiology which approach their limiting values, and thus suggest methods of control. For this reason the opportunity has been taken to examine specimens of the large cockroach *Macropanesthia rhinocerus* Sauss. which is found in the Mareeba-Dimbulula district and in some other localities in tropical and subtropical Queensland.

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The histology of this organism has been compared with that of other cockroaches, especially *Blattella germanica* (L.). Measurements of *Macropanesthia* and three other cockroaches are presented in Table 1. It will be noted that *Macropanesthia* weighs twelve times more than *Periplaneta americana*, and 320 times more than *Blattella germanica*. The linear dimensions of *Macropanesthia* are not great in comparison with those of many phasmids, or compared with the wing spread of many Lepidoptera, but by weight it ranks as a large insect, and it is bulk that is of the greatest consequence in the present discussion. *Macropanesthia* is certainly much larger than any of the insects mentioned by Rensch (1948) and it is, therefore, of value to examine whether his generalizations apply to this insect.

TABLE 1
APPROXIMATE DIMENSIONS OF VARIOUS COCKROACHES

Species	Weight (g.)	Length (cm.)	Maximum Width (cm.)	Width of Head (cm.)	Thickness of Abdomen (cm.)	Calculated Area (cm. ²)	Length of Gut (cm.)			Ratio Body Length to Gut Length
							Foregut	Midgut	Hindgut	
<i>Macropanesthia rhinocerus</i> Sauss.	19.5	7.5	3.5	1.1	1.4	—	3.1	11.0	12.0	3.5 : 1
<i>Periplaneta americana</i> (L.)	1.6 (0.91)†	4.0	1.3	0.6	0.6	>20.0§	1.8	1.8	2.5	1.5 : 1
<i>Cryptocercus punctulatus</i> Scudd.°	0.8	4.5	1.3	0.55	0.55	—	1.3	1.5	3.2	1.3 : 1
<i>Blattella germanica</i> (L.)	0.06 (0.04)†	1.1	0.5	0.2	0.2	2.0†	0.7	1.0	1.0	2.5 : 1

° Data from Cleveland *et al.* (1934) and from Gilmour (unpubl.).

† Data in brackets from Gunn (1935).

‡ Data from Simanton (1933). (It is not clear from the method whether wing area is included.)

§ From Richards and Anderson (1942). Figure excludes wings, head, appendages.

In discussing *M. rhinocerus*, Tepper (1894) notes that "this species is the largest and bulkiest Blattarid among Australian representatives so far as I am acquainted with them, and is probably not exceeded by any species elsewhere." It may therefore be the largest cockroach, living or extinct (see Carpenter 1947).

Little is known of the life history of *Macropanesthia*, but some details have been supplied by Mr. W. A. Henson, who has kindly collected the specimens on which this work is based. The roaches are infrequently observed during the dry season from March to October. They burrow quite deeply, about two feet below the surface of the sandy soil in stands of cypress pine (*Callitris* sp.). They make a nest of dead leaves, grass roots, etc., frequently among the pine roots. The young nymphs rarely appear above ground, but following rain the adults burrow to the surface, especially at night. They are most frequently found when they have fallen on to their backs and are unable to

right themselves. This species is also found in the brigalow (*Acacia harpophylla*) scrub about 70 miles west of Rockhampton, Queensland, and on Fraser Island off the coast of Queensland.

II. MATERIAL AND METHODS

Live specimens were received by air from northern Queensland in February 1948 (three specimens) and February 1949 (three specimens). Three males weighed upon arrival 19.5, 19.5, and 18.4 g. respectively, and one female weighed 19.5 g. This female had spermatozoa in the spermatheca, and it therefore seems likely that the specimens were adult, especially since no larger specimens exist in any collection known to us. The tissues of six specimens were fixed in Bouin's fixative or the alcoholic modification of it, and 5 μ and 10 μ sections were stained by Bodian's protargol method or by Heidenhain's azan method. The tissues of a number of *Blattella germanica* for comparison were treated in the same manner. Photomicrographs were taken with a Zeiss photomicrographic attachment, mostly with a 96 x oil immersion lens.

III. OBSERVATIONS

The results of this study are presented in a series of photomicrographs (Plates 1-4). The tissues will be briefly discussed in the same sequence.

(a) Cuticle

The cuticle of *Macropanesthia* varies markedly in thickness in different regions of the body, as shown in the following measurements:

	Average Thickness of Cuticle (microns)
Fore tibia	150
Pronotum	100
Fore coxa	90
Abdominal sternite	80
Abdominal tergite	45 (Plate 1, Fig. 2)

Richards and Anderson (1942) report that the pronotum of *Periplaneta* averages 40 μ , and the abdominal tergites of *Blattella* (Plate 1, Fig. 1) approximate 5 μ in thickness, seven times less than a comparable region in *Macropanesthia*. Nevertheless, the cuticle is divisible into the same regions in *Macropanesthia* and *Blattella*, although the vertical striations and pore canals in the cuticle of the former species are unusually clear. The hypodermal cells of *Blattella* are slightly larger than those of *Macropanesthia*.

(b) Muscle

The structure of the muscles of *Blattella* and *Macropanesthia* is very similar (Plate 1, Figs. 3 and 4). All described divisions of the fibril can be clearly seen in both, their striations are of comparable width, their nuclei are similar in size, and their tracheation and method of insertion are alike.

It has been claimed by Székessy (1947) that, in general, the slower the contraction of a muscle, the wider its striations. The majority of measurements of the muscles of *Macropanesthia* would appear to substantiate this, but the very narrow striation width of the muscles of the lateral oviduct was unexpected.

	Average Width of Striations of Muscles (microns)
Midgut longitudinal muscles	12.5
Thoracic tergosternal muscles	8.0
Hindgut circular muscles	7.0
Ventral abdominal muscles	6.5
Crop longitudinal muscles	6.0
Mandibular muscles	6.0
Dorsal vessel muscles	4.0
Muscles of lateral oviduct	2.0

Comparison between most muscles of *Blattella* and *Macropanesthia* reveals remarkably little difference between the two species. However, the width of the striations of the *Blattella* thoracic tergosternal is $6.5\ \mu$ compared with $8.0\ \mu$ for *Macropanesthia*. This difference is probably due to the fact that the muscle of *Blattella* is concerned with wing movement in this species.

Rensch (1948) describes the "parcelling" of muscle fibres in large insects and the development of intramuscular folds in very large species, but no such folds are found in *Macropanesthia*.

(c) Blood

In smears the haemocytes of *Macropanesthia* and of *Blattella* are almost indistinguishable. Ermin (1939) described three main cell types in the blood of *Periplaneta americana*, and these have all been observed in the blood of both *Blattella* and *Macropanesthia*.

(d) Dorsal Vessel

In spite of the increased work to be performed by the *Macropanesthia* heart in comparison with that of *Blattella*, there appears to be no striking difference between the two. The lumen of the *Macropanesthia* heart is considerably larger than that of the *Blattella* heart, but the difference in the thickness of the walls is quite small.

(e) Tracheal System

Nothing unusual has been noted in the tracheal system of *Macropanesthia*. It is, of course, well developed and the large tracheal trunks reach considerable dimensions (about 0.8 mm. diameter near the second abdominal spiracle), but the system is quite comparable with that of *Blattella* (Haber 1926). Small tracheal dilations containing taenidia are found, but they are not conspicuous. The abdominal spiracles are atriate with a lip type of closing apparatus, the lip being covered with short hairs. The external opening of the spiracle is well hidden by the projecting terga and sterna.

Respiratory movements of the body are scarcely visible, but ventilation does occur, and when an insect is disturbed it produces an unusual hissing noise resulting from a sudden expiration.

(f) *Nervous System*

Brain size in relation to head size in both *Macropanesthia* and *Blattella* is shown in Figure 1. The *Macropanesthia* brain is about 2 mm. in width, whereas that of *Blattella* is 0.7 mm. in width, but the *Blattella* brain occupies about ten times as much of the cranial cavity as does that of *Macropanesthia*. In spite of the increased bulk of the *Macropanesthia* brain the diameter of the intracellular tracheae is not proportionally greater than in the *Blattella* brain. The perilemma of *Macropanesthia* is much more conspicuous than that of *Blattella*. There is no marked difference between the size of the nerve cell bodies of the two species (Plate 1, Figs. 5 and 6).

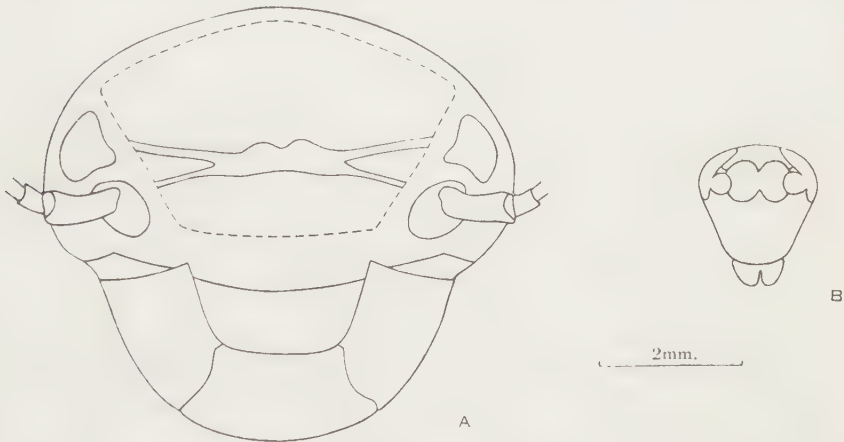


Fig. 1.—Diagrams of heads and supra-oesophageal ganglia of *Macropanesthia* (A) and *Blattella* (B), showing relative sizes.

Goossen (1949) has presented data on the relative sizes of the corpora pedunculata and central bodies of small and large insects. *Macropanesthia* does not follow the rule that the relative size of the corpora pedunculata is greater in large than in related small species, but the generalization that there is an inverse relationship between the size of the corpora pedunculata and the central body is true for *Macropanesthia*. Figure 2, made from tracings of several photomicrographs, shows the relative sizes of these structures. Calculated as percentage of half the maximum width of the protocerebrum, the index employed by Goossen (1949) and Rensch (1948), the figures for *Macropanesthia* are 34 and 40 per cent. for the corpora pedunculata and central body respectively.

The largest cell bodies in the nervous system (averaging $35\ \mu$ in diameter) have the "sponge-like outer zone" mentioned by Rensch (1948), as characteristic of very large animals.

The ommatidia of the two species in section appear remarkably similar, the only difference being in the slightly larger size of the pigment granules in *Macropanesthia*.

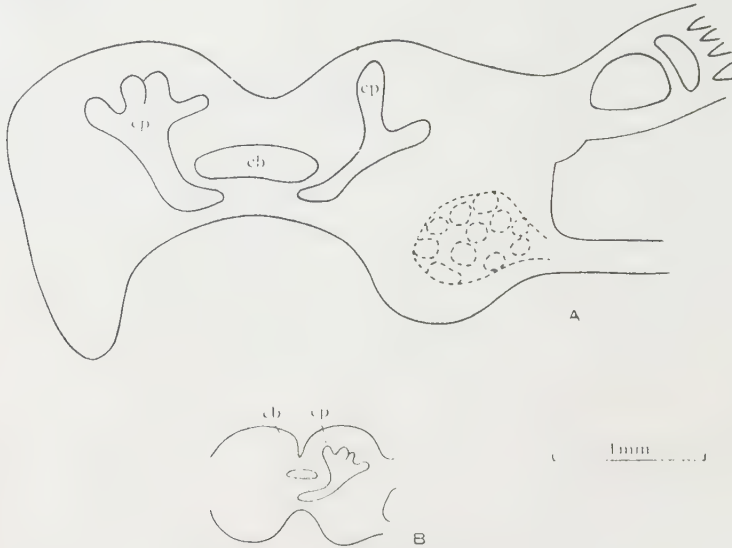


Fig. 2.—Diagrams of supra-oesophageal ganglia of *Macropanesthia* (A) and *Blattella* (B), showing relative sizes of corpora pedunculata (*cp*) and central bodies (*cb*). Outlined from photomicrographs of several frontal sections. Drawn to the same scale.

The diameter of the ventral nerve cords of *Macropanesthia* average about $170\ \mu$, approximately the same as those of *Periplaneta* (Roeder 1948), but twice the diameter of the cords of *Blattella*. Giant fibres are present in *Periplaneta* (averaging about $40\ \mu$ in diameter) and in *Blattella* (averaging about $15\ \mu$ in diameter). In *Macropanesthia* there are about 30 fibres of diameters between $7\ \mu$ and $14\ \mu$. None exceeds the latter measurement, but there are many fibres less than $7\ \mu$ in diameter. While the largest of these are only slightly smaller than the giant fibres of *Blattella* they are much smaller in comparison with the diameter of the cord. Thus the six largest fibres of *Blattella* occupy 10 per cent. of the cross-sectional area of the cord, whereas the 16 largest fibres of *Macropanesthia* occupy about 6 per cent. of the area, and the six largest fibres only about 3 per cent. Since the giant fibres have been shown to be fast fibres, the absence of large fibres is probably related to the slow reactions of *Macropanesthia*.

(g) Glandular System

The corpora allata of *Macropanesthia* (Plate 2, Fig. 8) are considerably larger than those of *Blattella* (Plate 2, Fig. 7), and this increase is accompanied by an increase in size of the central lumen. The latter is just perceptible in *Blattella* but conspicuous in the larger species. However, the cells are, if anything, smaller in *Macropanesthia*, a characteristic particularly evident

at the periphery of the gland. The nervous component of the corpora cardiaca of *Macropanesthia* is somewhat more conspicuous than the same tissue in *Blattella*, but histologically these glands of the two species are similar.

(h) Salivary Glands

The salivary glands of *Macropanesthia* (Plate 2, Fig. 10) and *Blattella* (Plate 2, Fig. 9) are morphologically similar although the former are considerably larger. Histologically the acini are slightly larger in *Macropanesthia* and the nuclei of the parietal cells are definitely larger (average maximum diameter in Bouin fixed paraffin sections $9\ \mu$ in *Macropanesthia* and $5\ \mu$ in *Blattella*). The ovoid nuclei of the central "zymogenic" cells, however, show less conspicuous differences and the intracellular ductules are similar in both species.

The salivary reservoirs of the two species are histologically similar, but morphologically are of much greater size in *Macropanesthia*, extending as far back as 2.5 cm. into the thorax when distended by fluid.

(i) Alimentary Canal

The gross morphology of the *Macropanesthia* gut is very like that of *Panesthia* as illustrated by Cleveland *et al.* (1934). The gut of *Blattella* has been illustrated by Snodgrass (1944). The oesophagus of *Macropanesthia* (Plate 2, Fig. 12) is lined by a thick ($20\ \mu$) chitinous intima densely covered with fine, caudally-directed spines. The intima of *Blattella* is thin (less than $2\ \mu$) and bears no spines (Plate 2, Fig. 11). The oesophageal epithelial cells of the two species differ only in that the cells of *Macropanesthia* are much more closely arranged, and as a result some of the nuclei are oppressed and ovoid. The muscularis of the two species is similar.

In the crop the intima is again thicker in *Macropanesthia* than in *Blattella*. This organ is much better supplied with muscles in *Macropanesthia* and the epithelium is less convoluted, rendering the organ less capable of enlargement, and thus of functioning as a storage organ.

The proventriculus, which in *Blattella* is conspicuous (Judd 1948) is completely absent in *Macropanesthia*. The oesophageal invagination of both species is similar.

Sections of the anterior end of the midgut show a most unexpected similarity in the two species (Plate 3, Figs. 15 and 16). The argentophil inclusions of the epithelium of *Macropanesthia* are coarser and extend to the base of the striated border; the connective tissue between the epithelium and the muscularis is more pronounced; but, apart from these relatively minor differences, the similarity between the tissues is very remarkable.

The cells of the *Macropanesthia* caecal epithelium (Plate 3, Fig. 14) are larger than those of *Blattella* (Plate 3, Fig. 13). The connective tissue layer is conspicuous and the base of epithelium is indented by it (Plate 3, Fig. 16). In *Blattella* there is some folding of the lumen margin of the epithelium but it is produced by the increase in cytoplasmic volume of the older cells, not by the folding of the epithelium itself.

In the region of the midgut caudal to the argentophil inclusions the epithelium of *Macropanesthia* is quite markedly taller ($110\ \mu$) than that of *Blattella* ($60\ \mu$). Also the cells are wider, averaging about $10\ \mu$ in *Macropanesthia* in comparison with $7\ \mu$ in *Blattella*. Conspicuous inclusions occur in a band just beneath the striated border in *Macropanesthia*, but are not found in *Blattella*.

The ratio of the diameters of the midguts of the two species is 1:3, while the ratio of the lengths of the same region is 1:11. The *Macropanesthia* midgut contains about 40 times as many columnar epithelial cells as the *Blattella* midgut.

In *Blattella* the hindgut epithelium is composed of two types of cells. There are patches of cuboidal cells (Plate 3, Fig. 17) connected by regions in which the epithelium is flattened. In *Macropanesthia* the epithelium is more uniform, and is composed mostly of cuboidal cells (Plate 3, Fig. 18), which are slightly smaller but have larger nuclei than those of *Blattella*. The muscularis in the larger species is more conspicuous and longitudinal muscles are abundant both inside and outside the circular muscle layer.

The rectal pads of both species are conspicuous, but they are considerably wider in *Macropanesthia* than in *Blattella*. In *Blattella* the pad is made up of about a dozen cells across its transverse dimension. These cells are about $200\ \mu$ in height and $12\ \mu$ broad (Plate 4, Fig. 19). In *Macropanesthia* there are about 100 cells in a transverse section of each pad, but the cells are actually smaller than those of *Blattella*, averaging $80\ \mu$ in height and about $20\ \mu$ in width (Plate 3, Fig. 20).

At the edge of each pad in both species is a curious modification of the cuticular lining. This takes the form of a rim around the pad at its junction with the cuboidal epithelium of small cells which join the pads.

Faecal pellets of *Macropanesthia* are barrel shaped and measure 10 mm. in length and about 5 mm. in diameter. They have six conspicuous ridges lighter in colour than the remainder and corresponding to the low epithelial tissue between the rectal pads. Their constancy of shape and the presence of these ridges indicates that they have been submitted to considerable pressure in the rectum. These pellets have over 300 times the volume of those of *Blattella* (length about 1.5 mm., diameter 0.7 mm.), a ratio very similar to the weight relationship between the two species.

(j) Malpighian Tubules

The malpighian tubules of both *Macropanesthia* and *Blattella* are very numerous. Their distribution in the body cavity seems to be similar in the two species, a conspicuous feature being the investing of the midgut caeca.

There is a marked difference between the sizes of the tubules. In *Blattella* (Plate 4, Fig. 21) they average about $30\ \mu$ in diameter with the epithelium about $10\ \mu$ in thickness and the lumen approximating $10\ \mu$ in diameter. In *Macropanesthia* (Plate 4, Fig. 22) the tubules are about $75\ \mu$ in diameter with the epithelium 20 to $25\ \mu$ in thickness and the lumen about $30\ \mu$ in diameter.

The same number of cells (4-6) surrounds the lumen in both species; and the cells of *Macropanesthia* are much larger than those of *Blattella*.

Near the entrance of malpighian tubules into the gut the striated border in *Macropanesthia* becomes very conspicuous and ciliform, but over the greater part of the tubules the border is similar in both species.

(k) Fat Body

The fat body of the two species is very similar, but the bacteroids of *Macropanesthia* are somewhat smaller than those of *Blattella*, and the nuclei of the *Macropanesthia* cells are larger (8.0 μ diam.) than those of *Blattella* (6.0 μ diam.).

(l) Connective Tissue

Connective tissue is better developed in *Macropanesthia* than in *Blattella*. This has been mentioned in relation to the gut, but it is also conspicuous in other locations as, for example, in the hypopharynx (Plate 4, Fig. 23) where it is well developed immediately beneath the thick epithelium of sensory cells at the apex of the organ. However, the degree of development of the connective tissue is not directly correlated with the size of the insect, since in the cockroach *Polyzosteria limbata* Burmeister (weight 1.5 g.) it is more conspicuous surrounding the midgut than it is in *Macropanesthia*.

(m) Pericardial Nephrocytes

The pericardial nephrocytes of *Macropanesthia* are larger (25 to 30 μ) than those of *Blattella* (15 to 20 μ), and in addition they contain many more conspicuous granular inclusions.

(n) Reproductive System

Mature gonads of either sex were not found in *Macropanesthia* but spermatheca containing sperm were present in one female, and immature ovaries in another, and accessory glands were found in one male. The latter were composed of tall columnar cells about 120 μ in height, compared with 35 μ for similar cells in *Blattella*.

(o) Other Tissue

A series of unusual bodies located in various parts of the *Macropanesthia* haemocoel should be mentioned. These appear to be groups of haemocytes which have formed a compact tissue (Plate 4, Fig. 24). Similar aggregations had been noted among the lobes of *Blattella* salivary glands, but in *Macropanesthia* they are much larger and more conspicuous. Moreover, they have been found near the dorsal vessel, beneath the ventral diaphragm, around the ventral nerve cord, and would appear to be formed anywhere in the haemocoel. Their function is unknown.

(p) Nuclear Size

The nuclei of different tissues of *Macropanesthia* differ considerably in size, as indeed they do in most insects. The majority of *Macropanesthia* nuclei are spherical or ovoid, and have a diameter in sectional material of about

8.0 μ . Most of the nuclei of the gut, salivary glands, blood, connective tissue, nervous tissue, and fat body fall into this category. But many nuclei are larger (diameters averaging about 12 μ) as in some malpighian tubules and in some nerve cell bodies. On the other hand, many are below average, as in many blood cells, neuroglia, some salivary gland cells, pericardial nephrocytes, and hypodermis; and in some tissues the nuclei are irregular in shape, being greatly flattened or elongate, as in muscles, male accessory glands, and some nervous elements. Nuclei of young *Macropanesthia* oocytes average 50 μ in diameter.

IV. DISCUSSION

(a) *The Effect of Size on Cell Structure*

The tissues described above fall into two groups; those in which there is a marked similarity between the tissues of *Macropanesthia* and *Blattella*, and those in which there are more or less marked differences. The first group, in which the tissues are histologically similar, includes muscle, blood, fat body, nervous tissue, the fore part of the midgut, the hindgut, and the ommatidia. The second group includes the cuticle, the malpighian tubules, the oesophagus, the hind part of the midgut, and the rectal glands. There does not appear to be any particular reason why a tissue belongs to one or the other of these groups. Rensch (1948) concluded that the differences in cell size between large and small organisms are small in those tissues in which cell division continues after the animal attains maturity, but this generalization does not appear to apply to a number of the above examples. Thus cells of the blood and midgut continue to divide, while those of the muscle, nervous tissue, and hindgut do not. On the other hand the cells of the hind part of the midgut continue to divide and yet are markedly different in the two species.

The generalization that in larger insects the cells are more slender (Rensch 1948, p.223) holds with the *Macropanesthia* midgut and male accessory glands, but not with most other tissues.

Haller's rule, that large species have relatively smaller brains than related smaller species, is well shown in Figure 1, but the corpora pedunculata of *Macropanesthia* are not as well developed as might be expected from the size of the organism, and are proportionally much less developed than those of *Periplaneta* (see Hanström 1940). The central body of *Macropanesthia* is relatively large, confirming Rensch's opinion that there is an inverse relation between the size of the corpora pedunculata and the central body.

(b) *The Limiting Factors to Insect Size*

Some of the factors which may affect the upper limits of the size of insects will now be considered in an attempt to determine whether *Macropanesthia* has approached these limits. These factors are (i) gravity, (ii) diffusion, (iii) absorption, (iv) thermal considerations, and (v) rate of conduction by nerves. Details of the implications of these factors have been published by Haldane (1927) and by Thompson (1942).

(i) *Gravity*.—The problems of locomotion imposed by the force of gravity are to a large part circumvented by *Macropanesthia*. It is wingless but, further, the weight of the insect is mainly in the abdomen which is not supported, but normally rests on the ground. The burrowing habit of the insect greatly reduces the effectiveness of gravity in limiting size. The legs are of considerable dimensions. The coxae of the fore-, mid-, and hindlegs are respectively 6 mm., 5 mm., and 5 mm. thick, and 7 mm., 10 mm., and 10 mm. wide at their widest parts.

The thickness of the cuticle is a specialization correlated both with the burrowing habit and with the necessity for the provision of insertions of considerable rigidity for the attachment of the muscles, particularly the leg muscles. But the limitations, stressed by Thompson (1942, p.52) imposed on size by the possession of an exoskeleton have not been reached in *Macropanesthia*, since an exoskeleton still serves the much larger land crabs.

(ii) *Diffusion*.—Krogh (1920) has concluded on the basis of theoretical considerations of insect respiration that the nymphs of Carboniferous dragonflies could not have had a completely closed tracheal system like the present-day representatives of the group, but that they must have possessed an open tracheal system and mechanical respiration. With the latter type of respiration, diffusion is the mechanism of gas exchange only in the non-ventilated tracheal branches. Insects can therefore reach considerable dimensions (much larger than are actually found) if their mechanism for tracheal ventilation is adequate. There is no doubt that ventilation occurs in *Macropanesthia*. Respiratory processes, therefore, probably do not limit the size of this insect.

(iii) *Absorption*.—The generalization that the gut is relatively longer in large than in small animals (see Rensch 1948, p.222) holds in a comparison between *Periplaneta* or *Blattella* and *Macropanesthia*, but not between *Periplaneta* and *Blattella* (Table 1). The food of *Macropanesthia* is not definitely known, but the gut of the specimens examined contained large quantities of vegetable matter. The hindgut in all specimens examined harboured a fauna consisting of a large species of amoeba, ciliates belonging to the Clevelandiidae, and nematodes.

The explanation of the generalization is undoubtedly to be found in the area of the gut available for secretion or absorption. It is therefore instructive to compare the area of the midgut of *Macropanesthia* with the secretive area in the human stomach. The area of the *Macropanesthia* midgut, excluding the caeca, approximates 880 sq. mm., that is, about 2.3 sq. cm. per g. of body weight. For man, the surface area of the glands of the mucous membrane approximates 2.7 sq. cm. per g. of body weight (data from Cowdry 1938, p. 404). This remarkable similarity suggests that convolution of the *Macropanesthia* midgut to increase the surface area may not be necessary if the epithelial cells of the two organisms are of comparable efficiency. The area of the *Blattella* midgut, excluding the caeca, approximates 5.2 sq. cm. per g. of body weight.

Data on the length of *Macropanesthia* malpighian tubules are not available, but they are probably not greatly different from Henson's (1944) figures

for *Blatta*, which indicate that there are about 300 cm. of excretory tubule per g. of body weight. The comparable figure for the larvae of the blowfly *Lucilia cuprina* is 250 cm. per g. (Waterhouse 1950). This suggests that the malpighian tubule is greatly inferior as an excretory mechanism to the human kidney, in which the capillary loops are about 7 cm. in length per g. of body weight (Cowdry loc. cit.).

It may be concluded from these calculations that the areas available for secretion and absorption have not approached the limiting values in *Macropanesthia*.

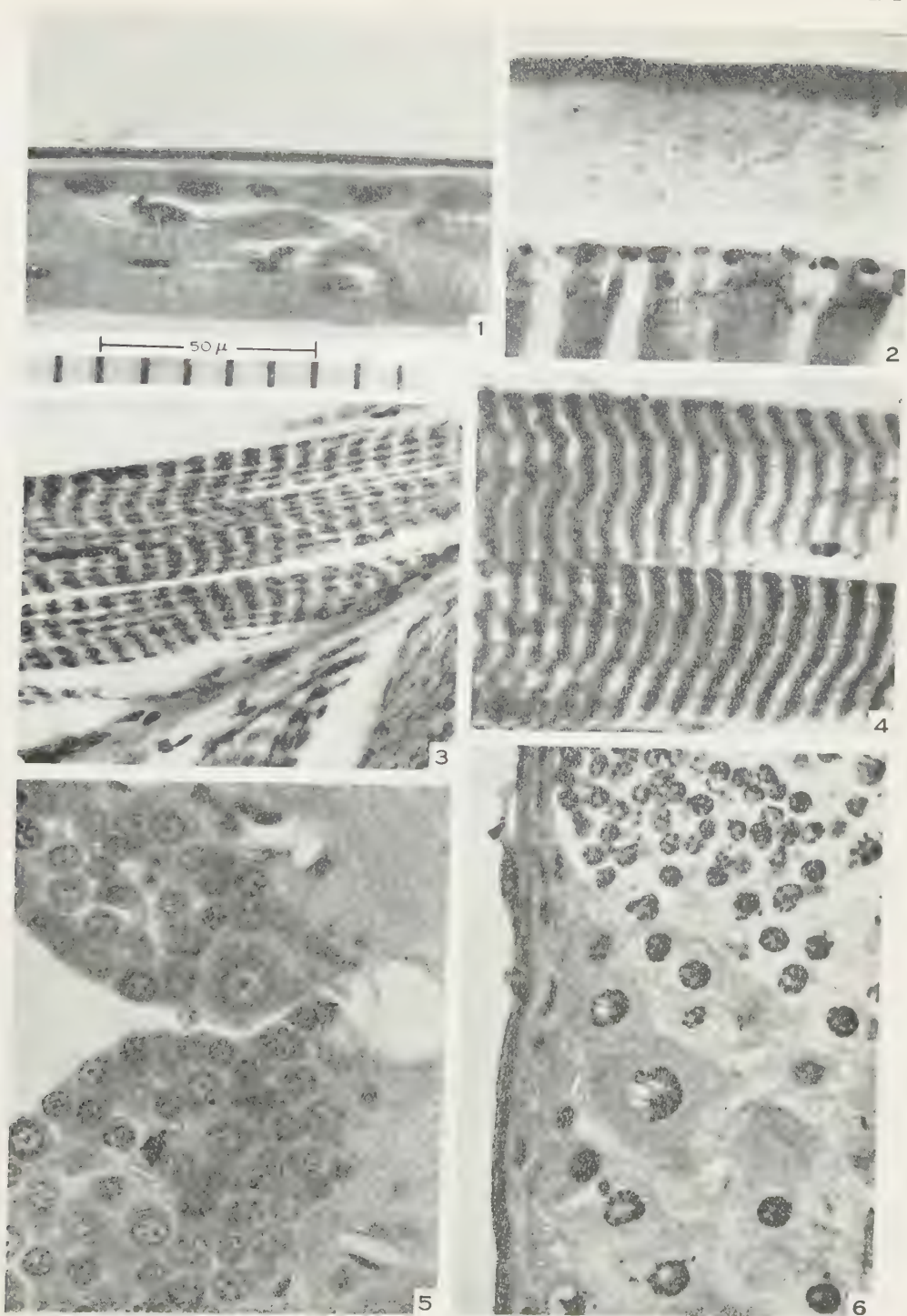
(iv) *Thermal Considerations*.—Bergman (1847) first indicated the effect of temperature on the distribution of animals and pointed out that small mammals are not found in the colder regions of the earth.

One of the most striking characteristics of large insects is that they are now practically confined to the tropics and that in past geological epochs they were apparently abundant in periods when the climate was probably tropical. This naturally tends to focus attention on temperature as a limiting factor in insect size, the more so since most other possibilities have been shown to be inoperative. The relation of body size to temperature has been investigated by Gumm (1942). He pointed out that a small animal is under a disadvantage in lowering its temperature since the water available for evaporation is limited. But it is very doubtful whether, in a relatively sluggish insect like *Macropanesthia*, heat production would prove an embarrassment. However, in such an insect the warming up following a cool period would place it in a distinctly disadvantageous position with respect to homoiothermic predators. It is unfortunate that no data are available on the temperature of blattids, but until evidence is produced against the hypothesis, it may be concluded that one of the factors limiting insect size is their inability to develop a homoiothermic mechanism.

It has been demonstrated that both the butterfly *Vanessa* and the bee *Bombus* are incapable of flight until they have raised their body temperature to about 30°C. (84°F.) (Krogh and Zeuthen 1941). Such an observation stresses the dependence of large insects on temperature and adds weight to the suggestion that this factor may well be important in the evolution of large insects.

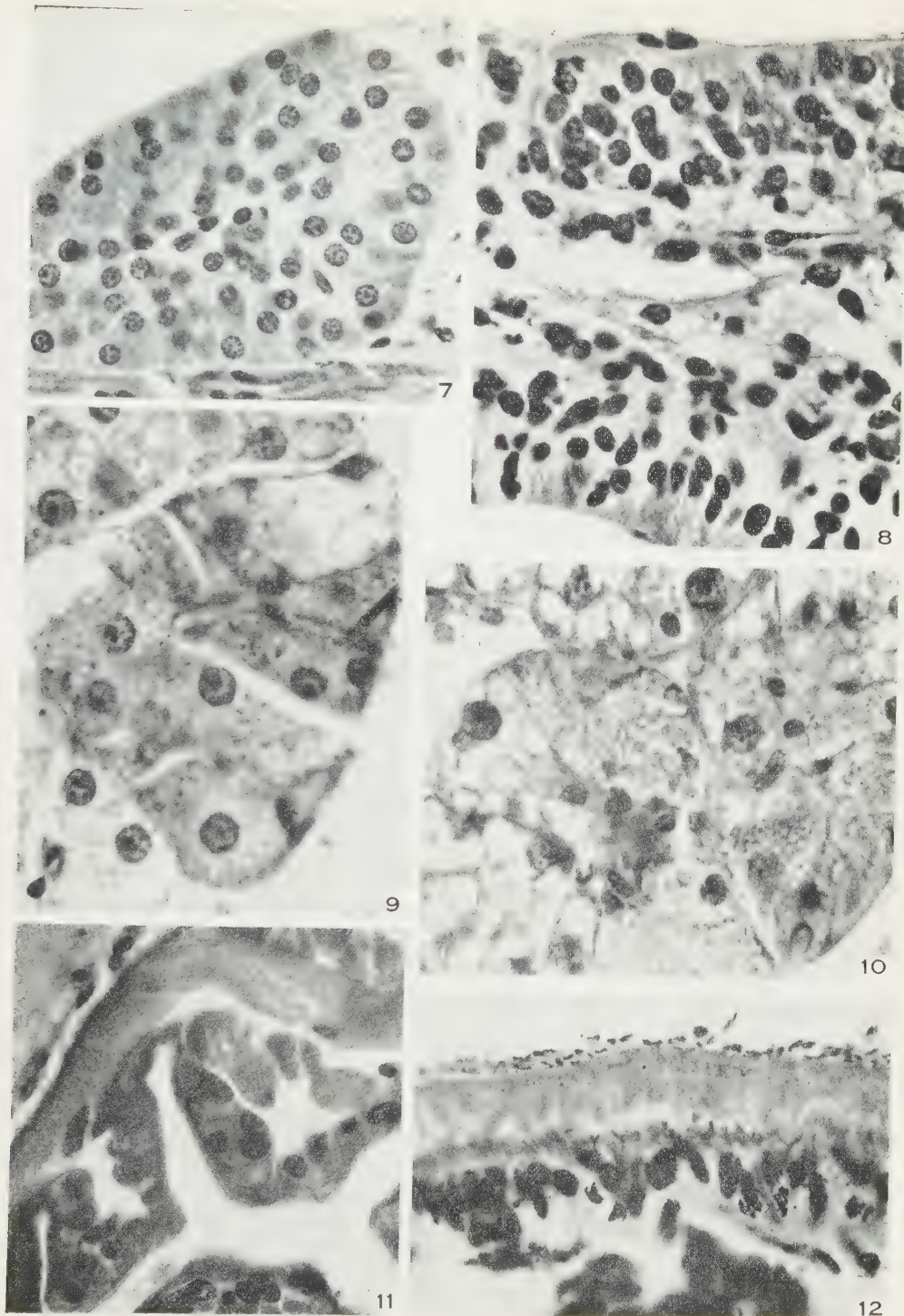
(v) *Rate of Conduction by Nerves*.—As pointed out above, the ventral nerve cord of *Macropanesthia* does not contain giant fibres. It is well known that the speed of conduction by a nerve fibre is correlated with its diameter, and the absence of giant fibres in *Macropanesthia* is reflected in the slowness of its reactions. It is interesting that increase in size in many large orthopteroids is accompanied by a decrease in the speed of reaction.* It is not possible to decide on the available evidence whether giant fibres are absent because the space in the ventral nerve cords is required for many smaller

* I am indebted to Dr. K. H. L. Key for descriptions of the behaviour of large acridoids, and for stimulating discussion on this subject.



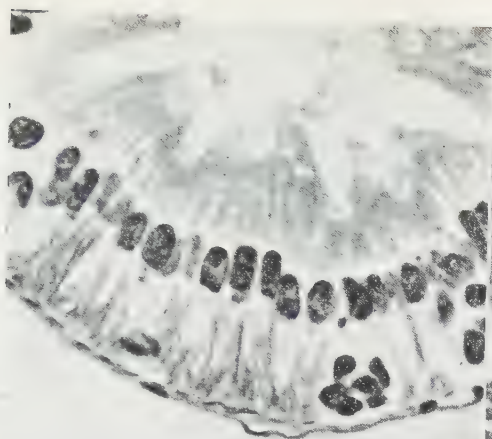
M. F. DAY.—THE HISTOLOGY OF A VERY LARGE INSECT, *MACROPANESTHIA RHINOCERUS* SAUSS. (BLATTIDAE)



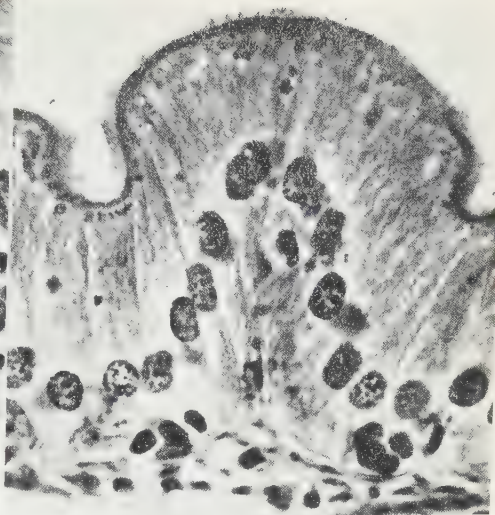


M. F. DAY.—THE HISTOLOGY OF A VERY LARGE INSECT, *MACROPANESTHIA RHINOCERUS* SAUSS. (BLATTIDAE)

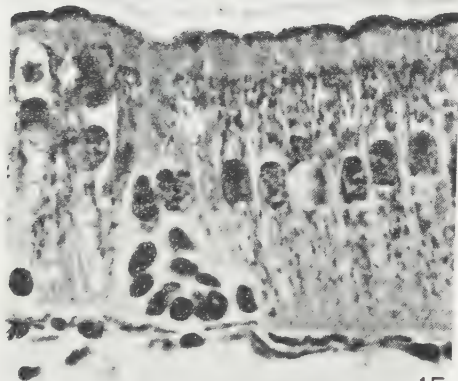




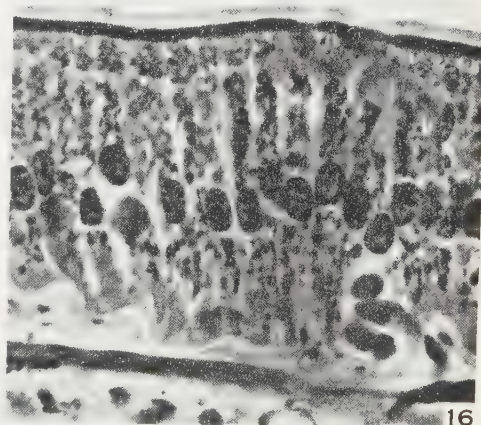
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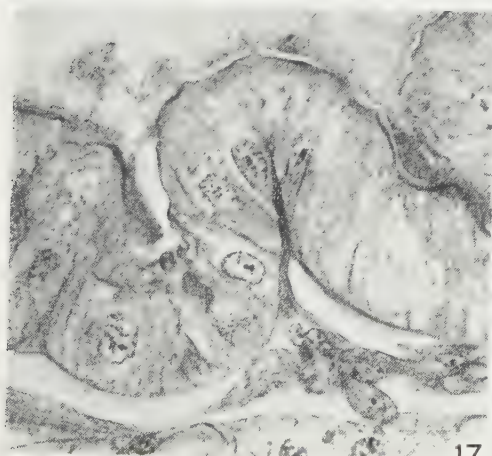
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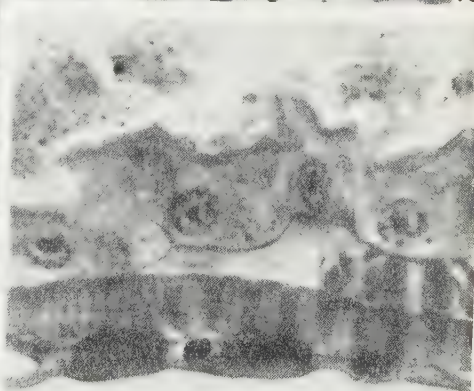
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M. F. DAY.—THE HISTOLOGY OF A VERY LARGE INSECT, *MACROPANESTHIA RHINOCERUS* SAUSS. (BLATTIDAE)



fibres as the organism becomes larger, or whether other factors determine the slower reaction speeds and this disposes of the need for fibres of large diameter. Although there would not appear to be any reason why the ventral nerve cords in large insects could not be increased in diameter, it is a fact that the slower speeds of reaction are characteristic of the large members of the group and that this would tend to place these larger insects at a disadvantage with respect to predators. Whether this would function in controlling the increase in size of insects cannot at present be decided. It is possible that the functioning of giant fibres would impose an impossible drain on the metabolic activity of very large insects.

V. CONCLUSIONS

Benedict (1936) found no characteristic of the physiology of the elephant which could be attributed solely to the large size of his experimental animal. And it is evident from the above observations that *Macropanesthia* shows few specializations in structure correlated with its size. Those specializations which are observed are more closely correlated with the environment of the insect than with its size. The absence of a proventriculus is the only unexpected anatomical characteristic. Of all of the factors influencing the size of animals considered by Thompson (1942), only those relating to temperature control and the rate of conduction of impulses by nerve fibres appear to be worthy of consideration as possibly limiting in *Macropanesthia*. It may be that metabolic factors may become limiting in large insects, as is suggested by extrapolation of the data of Gunn (1935) on *Blattella*, *Blatta*, and *Periplaneta*. However, the difference in size between *Periplaneta* and *Macropanesthia* is too great to validate such extrapolation. It is possible that factors other than those involved directly in the physiology of insects, as for example those involved in competition, may have played a major role in limiting the sizes of insects, including *Macropanesthia rhinocerus*.

VI. ACKNOWLEDGMENT

Thanks are due to Mr. W. A. Henson who kindly supplied the living *Macropanesthia* and much information about them.

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EXPLANATION OF PLATES 1-4

PLATE 1

Photomicrographs of tissues of *Blattella germanica* (left) and *Macropanesthia rhinocerus* (right). All Bodian preparations, same magnification (x 95 oil immersion x 8 ocular) except Figures 19 and 20.

Fig. 1.—*Blattella*. T.S. cuticle, epicuticle dark, endocuticle light. Note hypodermal cells and muscles. Beneath this is a 10 micron scale which applies to all figures except Plate 4, Figures 19 and 20.

Fig. 2.—*Macropanesthia*. T.S. cuticle. Details as in Figure 1. Note thick endocuticle.

Fig. 3.—*Blattella*. L.S. mandibular muscles.

Fig. 4.—*Macropanesthia*. L.S. mandibular muscles.

Fig. 5.—*Blattella*. T.S. brain in region of pars intercerebralis.

Fig. 6.—*Macropanesthia*. T.S. brain. Note thick perilemma and sizes of nerve cell bodies.

PLATE 2

(Details as in Plate 1)

- Fig. 7.—*Blattella*. T.S. corpus allatum. Note some differentiation between peripheral and central cells and indication of central lacuna.
- Fig. 8.—*Macropanesthia*. T.S. portion of corpus allatum. Note conspicuous central lacuna and marked differentiation into peripheral and central cells.
- Fig. 9.—*Blattella*. T.S. acinus of salivary gland. The parietal cells are smaller and more basophilic than the "zymogenic cells." One of the latter at the top right corner is vacuolate.
- Fig. 10.—*Macropanesthia*. T.S. acinus of salivary gland. Note differences in cytoplasm of different "zymogenic cells."
- Fig. 11.—*Blattella*. T.S. oesophagus as it penetrates between the sub- and supra-oesophageal ganglia. Note thin chitinous intima, epithelial cells with large nuclei, central longitudinal and outer circular muscles.
- Fig. 12.—*Macropanesthia*. T.S. small portion of oesophagus. Note very thick, spine-bearing chitinous intima and many small nuclei of epithelial cells.

PLATE 3

(Details as in Plate 1)

- Fig. 13.—*Blattella*. T.S. part of midgut caecum. The columnar epithelial cells have a somewhat crenulated inner margin, but the nuclei are all about equidistant from the basement membrane.
- Fig. 14.—*Macropanesthia*. T.S. part of midgut caecum. The epithelial cells are thrown into pronounced folds by the conspicuous development of connective tissue. Note granules in distal cytoplasm.
- Fig. 15.—*Blattella*. T.S. part of anterior end of midgut. Note regenerative nidus, distribution of conspicuous argentophil inclusions, and striated border.
- Fig. 16.—*Macropanesthia*. T.S. part of similar region of midgut. Note conspicuous development of connective tissue, large size, and distribution of argentophil inclusions.
- Fig. 17.—*Blattella*. T.S. part of hindgut. The cuboidal epithelium is in folds, permitting the lumen to increase in size.
- Fig. 18.—*Macropanesthia*. T.S. part of hindgut. The cuboidal epithelial cells are smaller than those of *Blattella*, but the musculature is much better developed. This section shows longitudinal muscles both within and outside the conspicuous band of circular muscles.

PLATE 4

(Details as in Plate 1, except that Figures 19 and 20 are at a lower magnification)

- Fig. 19.—*Blattella*. T.S. rectum. The contents have contracted during fixation. The six pads are joined by a poorly developed epithelium.
- Fig. 20.—*Macropanesthia*. T.S. portion of rectum, showing junction of two rectal pads. External longitudinal muscles are well developed between the pads. Each pad is very broad in comparison with those of *Blattella*.
- Fig. 21.—*Blattella*. T.S. group of malpighian tubules.
- Fig. 22.—*Macropanesthia*. T.S. single malpighian tubule. Note greater increase in size in comparison with tubule of *Blattella*.
- Fig. 23.—*Macropanesthia*. Connective tissue from hypopharynx. Haemocytes are scattered among the connective tissue strands.
- Fig. 24.—*Macropanesthia*. Section of part of organ of unknown nature located near ventral nerve cord in abdomen. Cells composing it have the appearance of loose aggregate of haemocytes.

STUDIES OF THE PHYSIOLOGY AND TOXICOLOGY OF BLOWFLIES

XIV. THE COMPOSITION, FORMATION, AND FATE OF THE GRANULES IN THE MALPIGHIAN TUBULES OF *LUCILIA CUPRINA* LARVAE

By D. F. WATERHOUSE*

[Manuscript received November 30, 1949]

Summary

Lucilia cuprina larvae possess two pairs of malpighian tubules. One pair, having a yellow appearance, is restricted to the posterior end of the body. The other pair commences at the posterior end of the body and runs forwards as far as the brain and then backwards on either side of the gut to enter it by a common duct. Whereas the ascending regions are greatly enlarged and have a white appearance caused by many small spherical granules in the lumen, the descending regions have the usual yellow appearance. The tubules are held in place at several points by fine strands of connective tissue.

A few small granules are present in the tubules of unhatched larvae. After hatching they increase rapidly in number and size and, when larvae have finished feeding, granules having a diameter up to about 16 μ are present. During pupal development the permeability of the cells of the granule-accumulating regions increases and granules are flushed down into the hindgut, where they contribute to the meconium to be excreted shortly after emergence of the adult. Although there still remains some differentiation of the tubules in the adult, granules do not accumulate unless a diet rich in calcium or magnesium salts is provided continuously.

Addition of calcium or magnesium to the larval diet leads to a great increase in granule accumulation and also to an increase in granule size (up to 20 μ). Addition of large amounts of phosphate depresses granule formation, presumably by binding calcium and magnesium in relatively insoluble form in the alimentary canal. Occasionally meat-fed larvae contain few or no granules.

The larval granules are soluble in dilute acids, and show birefringence, but no fluorescence. Analyses have shown that the granules contain large amounts of magnesium and calcium phosphates, some magnesium ammonium phosphate, large or small amounts of calcium carbonate, and a little magnesium carbonate. Both barium and strontium can be detected by a staining reaction and by spectrographic analysis in the granule-accumulating region. Magnesium and calcium are of about equal importance as granule constituents, although the composition of the granules is greatly influenced by the diet. On some diets, such as those extremely rich in calcium and relatively poor in phosphate, calcium carbonate became the preponderant constituent of the granules. Even under these circumstances the amount of respiratory carbon dioxide bound in granule form is less than 1 per cent. of that produced by the larvae.

Protein, sulphydryl, chloride, sulphate, oxalate, lactate, potassium, and iron have not been detected in the granules and uric acid cannot be present in more than traces. On the other hand, urates are present in the yellow

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regions of the larval tubules and occur as small granules in the lumen throughout the tubules in the late pupa and adult. Riboflavin, copper, and sulphydryl are present in the tubule cells. There is no difference in the pH and Eh of the contents of the granule-accumulating and yellow regions.

Although several different types of larval granule may be distinguished visually, staining reactions suggest that each granule is normally a heterogeneous mixture of several salts, either laid down simultaneously, or in successive concentric layers.

Two enzymes associated with sites of calcification in vertebrates and in many invertebrates, namely carbonic anhydrase and alkaline phosphatase, appear to play no part in granule formation.

The conclusion is reached that the granules are merely waste products, formed as a result of the specialized nature of the cells in the accumulating region and stored there by virtue of the absence of peristalsis and of adequate flushing of water down the tubules.

I. INTRODUCTION

Accumulations of granules are characteristically present in one pair of malpighian tubules of many dipterous larvae, a feature which has been recorded frequently since the early observations by Dutrochet (1818) of milky vessels in larvae of the syrphid *Eristalis tenax*. The presence of granules, conferring a conspicuous white appearance upon the particular region of the tubules containing them, has led to much speculation, but to very little accurate information, upon their composition and function. Whereas some authors carried out no chemical tests whatever, a number (Batelli 1879; Krüger 1926; Müller 1924; Pantel 1898, 1914; Roubaud 1913; Vaney 1900) recorded solution of the granules in dilute acid, accompanied by evolution of gas thereby demonstrating the presence of carbonate. Although it has always been assumed that calcium was combined with this carbonate to form the granules, the actual occurrence of calcium has only occasionally been demonstrated (Müller 1924; Pantel 1898; Roubaud 1913; Vaney 1900, 1902). Furthermore, while the occurrence of magnesium in the granules has been suggested on several occasions, there appears to be no recorded demonstration of its presence by chemical tests. The presence of calcium oxalate, phosphate, and urate in insect excreta has been recorded (Uvarov 1928; Wigglesworth 1939), but most reports are based on inadequate chemical evidence and there are no authentic records of the occurrence of these salts in the excreta of dipterous larvae. It is clear, therefore, that there is a need for accurate determinations of the identity and relative importance of the materials present in the granules. Furthermore, there is no information available on the factors influencing granule accumulation.

Three suggestions have been made in the literature regarding the function of these granules. They have been regarded as serving as a reserve for calcium (Vaney 1900, 1902), as having a respiratory function by virtue of the carbon dioxide they remove from the body (Keilin 1921), or as being merely an ordinary product of excretion (Pantel 1914). Certain specialized uses to which

accumulated granules are put are known in some dipterous families, such as the strengthening of the larval (Müller 1924) or puparial cuticle (Keilin 1921; Schutte 1921).

It will be shown below that, in *L. cuprina*, the granules represent merely a temporary storage of waste excretory products, amongst them respiratory carbonates, which are transferred from the tubules to the hindgut in the pupal stage to be excreted by the newly emerged adult.

II. METHODS

(a) Biological

L. cuprina larvae were bred under aseptic conditions at 30°C. on a medium consisting of 88 per cent. egg white, 11.7 per cent. dried baker's yeast, and 0.3 per cent. sodium chloride (Lennox 1939).

To obtain samples for chemical analysis, larvae were dissected under the least possible quantity of saline (Ephrussi and Beadle 1936) and the various tissues separated out on to small, previously weighed, pieces of coverslip. Identical paired samples of the granule-containing portions of the malpighian tubules were obtained by accumulating the granules from one of the two storage tubules of each larva on one coverslip and from the other on a second coverslip. Blood was collected in the manner already described (Waterhouse 1945b).

(b) Chemical

Inorganic calcium, magnesium, and phosphate* were extracted by treatment of the dried tissues with 20 per cent. trichloroacetic acid for one hour at 37°C.

Phosphate was estimated colorimetrically by the method of Fiske and Subbarow (1925). Calcium was precipitated as oxalate after adjusting the pH to between 5 and 6 with ammonia, thereby reducing the danger of magnesium being occluded in the calcium precipitate. After centrifuging, the precipitate was washed with 2 per cent. ammonia in equal parts of ethyl alcohol, ether, and water (Wang 1935) and centrifuged again. The oxalate was then estimated by titration with permanganate. Magnesium was precipitated as the ammonium phosphate. This was washed in the same way as the calcium precipitate and its phosphate content estimated colorimetrically.

When information was required on total mineral content, the tissues were digested on a sand bath with concentrated sulphuric and nitric acids, or concentrated perchloric and nitric acids.

For the determination of carbonate, the volume of carbon dioxide liberated at 37°C. following treatment with 0.5N hydrochloric acid was measured manometrically. Ammonia was determined colorimetrically after the addition of Nessler's reagent to an acetic acid extract of the granule-accumulating regions.

* Although this is termed inorganic phosphate it includes esters (Umbreit, Burris, and Stauffer 1947).

III. THE LARVAL MALPIGHIAN TUBULES

In *L. cuprina*, as in other calliphorid larvae, there are two pairs of tubules, each pair opening into a short common duct leading into the alimentary canal (Fig. 1). One pair of tubules, that discharging into the left side of the alimentary canal, commences posteriorly and runs forward, one tubule on each side of the body, to about the level of the brain before turning sharply and running back down on either side of the gut to enter it at the end of the midgut. This turning point near the brain, which is designated as the "bend" region for convenience of later description, marks a rapid transition in the appearance of the tubules. The cells of the descending arms have the general appearance of a string of beads, the individual cells protruding into the body cavity. They are deep orange-yellow and contain many granular inclusions, but there is no solid material in the lumen. In sharp contrast, the cells of the ascending arms are far smoother in outline, they contain relatively few inclusions which are generally of a yellowish-brown colour and the lumen of the tubule is greatly enlarged, being stuffed with minute spherical granules (Plate 1, Figs. 1, 2, 5).

The second pair of tubules, discharging into the right side of the alimentary canal, are uniform throughout their entire length and closely resemble in appearance the descending arms of the pair just described. The two component tubules run backwards to the posterior end by a very winding course, their blind ends being joined by connective tissue to the respective blind ends of the anterior pair of tubules (Plate 1, Fig. 4). As a rule, the posterior pair of tubules contains no granules. The two short ducts by which the two pairs of tubules discharge into the gut are colourless and are formed of cells which do not protrude into the haemocoel. In larvae 2.5 days old, about 10 mm. in length, and of average weight 30 mg., each anterior tubule is about 20 mm. long and each posterior tubule about 16 mm.

The tubules are generally well tracheated, with the exception of the posterior two-thirds of the granule-accumulating regions which are comparatively poorly supplied. The tracheae are largely responsible for maintaining the tubules in position within the larva, although they are assisted in this by strands of connective tissue which are inserted in the body wall, the alary muscles or the midgut caeca. These strands of connective tissue (Fig. 1; Plate 1, Fig. 4) occur at the bend region, at the posterior end of the tubules, and at three intermediate positions (Waterhouse, unpublished data).

The histology of the tubules presents no unusual features. The yellow regions (Plate 1, Fig. 6) have an outer basement membrane, a rather uniform cytoplasm, and a distinct inner border, which is usually striated, but seldom appears free. Many of the granules which can be seen within the cells of living tubules are not visible in sections, having been dissolved by the fixatives. At times small vesicles of material can be seen protruding through the cell border, but these are probably fixation artefacts or degenerating cells (Wigglesworth 1931). The lumen of the tubules is usually bounded by two or three cells with rather indistinct boundaries.

The granule-accumulating region differs principally in the enormous distension of the lumen to hold granules. The cells are extremely flattened and thin, so that fine details are difficult to see. The narrow layer of cytoplasm is apparently uniform and basement membrane and inner cell border are very thin.

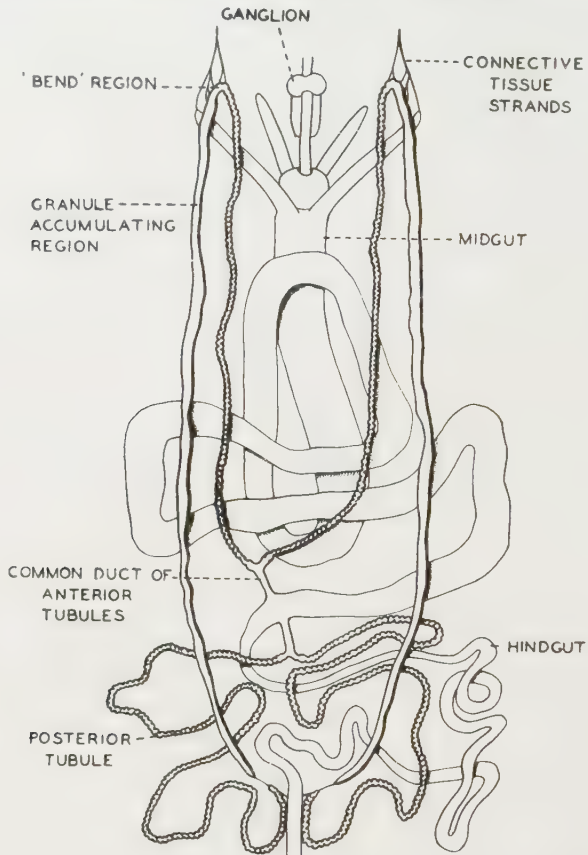


Fig. 1.—Diagram showing disposition of malpighian tubules in *L. cuprina* larvae.

The lumen of the common duct is bounded by several cells. Inner circular muscles and outer longitudinal muscles invest these cells. Their haemocoel boundary is richly supplied with tracheae and tracheoles which, however, do not become intracellular as they do in the adjoining midgut.

The granules of the accumulating region are transparent and colourless when viewed with transmitted light, but white by reflected light. They are generally spherical and, in larvae 2.5 days old on the control medium, range in diameter from $0.5\ \mu$ or less to about $10\ \mu$, with the bulk of the materials in granules of 4 to $8\ \mu$ diameter. When mounted in balsam and examined under oil immersion a number of different types of granule can be clearly distinguished (Fig. 2; Plate 1, Figs. 1, 2, 5).

For instance, granules may have a uniform transparent interior; they may possess one or more centrally located dark granules, spherical or irregular in shape; they may possess one or more uniform or granular internal layers of different light-transmitting properties from the bulk of the granule; or they may be entirely composed of fine granular material. Occasionally irregular granules, which have been formed by the fusion of two or three smaller granules, may be seen. All of these types may have an indistinct or a distinct outer boundary, indicating different refractive indices of the surface layer.

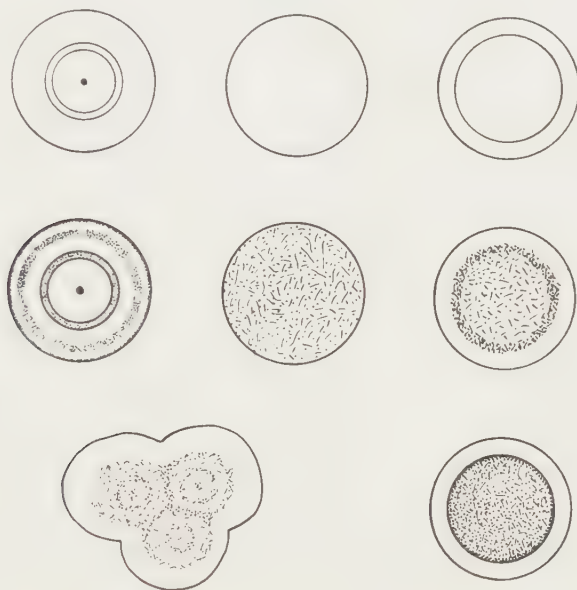


Fig. 2.—Diagrammatic representation of some common types of granule.

IV. FORMATION AND FATE OF GRANULES IN THE TUBULES

(a) *Fully Developed Eggs*

Dissection of larvae removed from the egg membranes shortly before hatching demonstrated that granules were already present in small numbers in the lumen of the ascending arms of the anterior pair of malpighian tubules. The granules covered a range of sizes, the largest had a diameter a little under $2\ \mu$, granules with a diameter of $1.8\ \mu$ were of frequent occurrence, and there were many with a diameter of $0.5\ \mu$ or less.

(b) *Larvae*

By the time larvae had fed on the artificial medium for a few hours, granules were clearly visible in large numbers in the ascending arms of the anterior pair of tubules. With increase in size and age, the granular deposits increased in quantity, and the size of the largest granules also increased rapidly.

When, after three or four days, larvae were ready to leave their food to pupate, spherical granules as large as $16\ \mu$ in diameter could be seen, with occasional irregular granules of somewhat greater width. Most, however, had a diameter of less than $10\ \mu$. There was some variation from one individual to another in the amount accumulated and also in the appearance of the granular region. In some larvae the cell outline was irregular, with protruding cells alternating with constricted regions. Where the cells protruded, the lumen was enlarged and stuffed with granules, while the constricted regions coincided with a narrow lumen with less granular material. In other larvae the narrower regions were absent and the diameter of both tubule and lumen relatively uniform. This latter state occurred most frequently when the tubules were stuffed with granules.

A day or two after larvae ceased feeding, a pale green coloration of the contents of the granular regions of the tubules became apparent. This coloration increased in intensity up to about pupation and then remained unchanged. It was most intense in the posterior third or quarter of the granular region, becoming progressively weaker towards the anterior end. The material responsible is insoluble in alcohol (absolute or 70 per cent.), chloroform, ether, carbon tetrachloride, and acetone. The coloration was no more intense in larvae fed on blood or liver than on the artificial medium. As might be expected, therefore, tests for biliverdin and urobilin were negative. The material responsible for the coloration is apparently some by-product of metabolism produced in the prepupal stage shortly prior to pupation and may be related to the production of the phenols involved in the hardening of the puparium. Immersion of the intact tubule in distilled water caused initially a breakdown of the cells, accompanied by a gradual reduction in the intensity of the green coloration. The pigment, therefore, is somewhat soluble in water.

(c) *Pupae*

During the early stages of metamorphosis the general arrangement of the tubules is little altered, the granules remaining in the ascending arms of the anterior pair and retaining the greenish coloration acquired in the prepupal period. After the tubules have become visibly associated with the developing adult alimentary canal, discharge of the granules into the hindgut commences. This discharge begins shortly after a salmon pigmentation has appeared in the eyes and also after the dorsal thoracic bristles and those of the head have commenced to darken. It does, however, precede the darkening of the remaining bristles. The process of transference is a gradual one, being substantially complete by the time of emergence although, in the freshly emerged adult, there are sometimes small aggregates of granules which have not been discharged from the tubules. These may be present either in the former granule-accumulating regions, or on their way down the yellow portions of the tubules.

The factors initiating the transference of the granules are of some interest. The common ducts of each pair of tubules, which are well supplied with

muscles as in *Calliphora* larvae (Eastham 1925), undergo frequent peristaltic movements, which lead to the discharge of their contents into the hindgut. These movements influence directly only the granules which are now present in the adjoining portion of the tubules and could scarcely bring about their transfer from the granule-accumulating region. No peristalsis of the individual tubules occurs and their only movements appear to be bodily displacements due to peristalsis of the alimentary canal or to locomotion. An attractive hypothesis is that the fluid intake of the granule-accumulating region alters at the time of granule transfer, so that the granules are flushed down the tubules, a circulation of water being achieved by its reabsorption in the hindgut.

Evidence strongly supporting this view comes from two sources. Just at the stage at which granules have commenced to move towards the hindgut, the latter is often seen to contain a green fluid, but only very few granules. This would occur if the first flushings of the granule region dislodged a few granules, removing at the same time some of the green pigment. It would also supply a satisfactory explanation of the simultaneous appearance of the first few granules and of the pigment solution in the hindgut.

The second line of evidence comes from the injection of saline saturated with indigocarmine. No dye is taken up by the granule-accumulating regions, although the yellow regions rapidly absorb it and concentrate it in the lumen. In pupae, on the other hand, rapid dye uptake by the granule-accumulating region does occur. This is true even before the yellow regions have regained their function following reorganization. As soon as the yellow regions become functional again they too take up dye and both granules and dye are discharged into the hindgut. It is clear therefore that, in the early pupal period, the permeability to dyes of the cells of the granule-accumulating region has altered considerably, and one might well expect this to be associated with a greater capacity to take up fluid. Further proof of altered permeability comes from the observation that urates, which are absent from the larval accumulating region, but not from the remainder of the tubules, begin to appear in quantity in the accumulating regions at this stage in pupal development (see later).

One further observation may have some bearing on granule transfer. After larvae have been immersed for several hours in water, the accumulating regions commence to lose their characteristic white appearance and become more transparent. Addition of hypertonic saline to dissected larvae causes a return of the tubules to their normal appearance, indicating that the greater transparency was due to the accumulation of fluid in the lumen of the tubules. Presumably, the water-reabsorbing process has been interfered with by oxygen lack more than the water-secreting process. A parallel observation has been recorded by Gamo, Yamaguchi, and Nagai (1933), who found that the permeability of the midgut of the silkworm larva altered considerably if the larvae were subjected to oxygen lack. Distension with fluid would tend to produce discharge of the granules and, while this could not be observed in control larvae, a small proportion of the granules were discharged from the massive

deposits accumulated by larvae fed on calcium-enriched diets (see Section VI (a)). The poorer supply of oxygen during metamorphosis might favour the secretion of water into the accumulating region at a greater rate than it could be reabsorbed and this would result in discharge of the granules.

The amount of granular meconium present in the hindgut, even before more than a small fraction of the accumulated granules has been discharged, often exceeds the total originally present in the granule-accumulating regions. It is apparent, therefore, that the anterior pair of tubules has been discharging both newly formed and pre-existing granules. This is also suggested by the observation that, at times, portions of the yellow regions contain predominantly very small granules, but only very few larger ones, which would also be present if the granules came from the accumulating regions. Some time after discharge of granules has commenced from the anterior tubules, numerous small granules also appear in the posterior pair and are discharged into the hindgut. These granules (Plate 1, Fig. 3) are roughly spherical in outline, but are quite different from the regular spherical granules accumulated during larval life.

The increased fluid uptake by the tubules thus appears to serve two functions, that of discharging larval excretory products stored in the tubules and that of removing excretory products accumulated elsewhere in the body during the early stages of metamorphosis. It will be shown later (Section V (n)) that large quantities of urates are taken up by the tubules during this period, appearing first in the lumen in the form of small granules and subsequently contributing to the meconium.

(d) *Adults*

Shortly after emergence, most of the contents of the hindgut are expelled as meconium and the tubules now contain few granules. The larval granule-accumulating regions of the tubules can still be distinguished by their light pigmentation, smaller diameter, and smaller volume of the cells.

When adults are fed on a diet of sugar and water, scattered granules can often be seen in the blind ends of both pairs of tubules, as well as along their length. These granules occur more frequently and in greater numbers in the anterior pair of tubules than in the posterior pair and are small and not as uniformly spherical as the larval granules. There are good reasons for believing that, in the adult, granules are continually being formed and discharged. Thus, on normal diets, they do not increase in size or in number with increasing age, and it is possible to observe granules at any one time in all stages of discharge.

V. REACTIONS OF THE GRANULES

(a) *Solubility*

Granules from the tubules of fully grown larvae dissolved rapidly in concentrated mineral acids and slowly in dilute acids (e.g. N/15 hydrochloric acid). They also dissolved in 1 per cent. trichloroacetic acid and in 1N acetic

acid. All types of granule appeared to dissolve at the same rate, so no separation of granules could be obtained by this means. Solution was accompanied by an evolution of gas, which was found by analysis to be carbon dioxide (see Section VIII). There is no doubt, therefore, that carbonates are present. Adult granules also dissolved in dilute acid with evolution of gas. Larval granules are not soluble in boiling water, alkalis, nor in any of the common organic solvents.

(b) *Density*

The density of the granules was determined by the float or sink method using tetrabromethane (sp. gr. 2.95) diluted with carbon tetrachloride (sp. gr. 1.59). The values obtained lay between 2.3 and 2.8.

(c) *Examination with Polarized Light*

The granules are distinctly birefringent (Plate 1, Fig. 5) showing under crossed polars a black cross reminiscent of starch grains and of the calcospherites of the celery fly *Acidia* (Keilin 1921). This black cross remains stationary when the granules are rotated, indicating that the micelles are oriented normally to the periphery. The amount of light transmitted and the birefringence produced vary from granule to granule, indicating that the granules are not homogeneous.

(d) *Examination with Ultraviolet Light*

When examined by ultraviolet light the granules showed no fluorescence; nor do undamaged tubules. Slight damage to the tubule cells, however, results in the diffusion into the surrounding fluid of a material having an intense yellow fluorescence. A chemical examination of tubules from liver-fed larvae has shown this material to be riboflavin. With approximately 1.5 γ per larva in the tubules, riboflavin appears to be present in a concentration greater even than that recorded by Metcalf (1943) for *Periplaneta*. Since riboflavin appears only in the immediate vicinity of damaged cells and since successive damage along the length of the tubules always produces fresh fluorescence, it is clear that the riboflavin is present within the cells and not in the lumen. Less fluorescent material is present in the cells of the granule-accumulating region than in the yellow regions and none is present in the common exit duct, except just at the junction of the two tubules. Since the intact tubules do not fluoresce, the riboflavin may be present in a reduced state. The pigment appears to be similar in many ways, although not in this respect, to the cytoplasmic form of riboflavin described in *Periplaneta* (Metcalf and Patton 1942; Metcalf 1943). Following the addition of large quantities of riboflavin to the diet (up to 60 γ per g.) the cells of the accumulating region sometimes show an orange fluorescence, whilst the remaining portions are unchanged. This may indicate that the oxidation-reduction potential is not as low in the cells of the accumulating region as elsewhere, although confirmation of this was not obtained in studies with redox dyes (see Section VIII(b)). In addition, the relatively poor tracheation of this region mentioned earlier (Section III) would not lead one to expect a higher Eh here than elsewhere.

(e) Phosphate

The cobalt sulphide visualization method for phosphate (Danielli 1946) was applied to sections of the accumulating regions and to smears of granules. An intense positive reaction was given by many of the granules (Plate 1, Fig. 1). Staining was generally uniform throughout the granule, but occasionally it was confined either to the central region or to the periphery. However, some granules of all types remained unstained so that, unless stained, phosphate-rich granules do not have a characteristic appearance. Sections of the yellow regions of the tubules did not stain for phosphate.

When granules from the calcium- and magnesium-enriched media shown in Table 7 were treated, only a small proportion of those from calcium-fed larvae and none of those from magnesium-fed larvae stained at all intensely. While it might be expected that the very large amounts of calcium carbonate present on the former diet would result either in many granules containing no phosphate, or granules in which any phosphate would be effectively masked, the absence of distinct staining of granules from magnesium-fed larvae where phosphate is the predominant anion in the granules cannot be explained.

(f) Sulphate

If sulphate is excreted by the granule-accumulating region the presence of calcium sulphate in the granules would be expected. Addition of barium chloride to a concentrated solution of the granules in dilute acetic acid produced no precipitate, indicating that sulphate was absent. Further, no reaction was given when the larval food contained large amounts of added sulphate. The granule-accumulating regions, therefore, are not involved in sulphate regulation.

(g) Chloride

The addition of silver nitrate to a concentrated solution of granules in dilute nitric acid did not produce a precipitate, indicating that chloride was absent.

(h) Calcium

Granules, smeared on a slide, were immersed for 10 minutes in a saturated aqueous solution of Gallamine blue which, according to Stock (1949) forms a specific purple lake with calcium. All, or almost all, of the granules became stained, the colour varying from very light to dark purple (Plate 1, Fig. 2). Sometimes inner inclusions or rings stained more deeply than outer layers of the granules and sometimes the reverse occurred. It appears that calcium is a component of all or almost all granules. Granules from larvae which had fed on media greatly enriched with magnesium and which contained far more magnesium than calcium (see Table 7) were found to stain very lightly or not at all. This provides good evidence that magnesium does not stain with Gallamine blue.

When dissected larvae were treated with the dye, the granule regions of the tubules stained purple, and the remainder of the body a non-specific blue.

(i) Magnesium

Titan yellow and Magneson II, dyes which produce characteristic absorption colours on magnesium hydroxide (B.D.H. 1939) were tested on dissected larvae made alkaline with dilute sodium hydroxide.

Titan yellow in aqueous solution produced the red coloration characteristic for magnesium in the yellow regions of the tubules even before addition of alkali, suggesting that the tubules have an alkaline reaction. After the addition of dilute alkali the granule region of the tubules and the cut edges of the cuticle also stained. The granules sometimes stained a light pink, sometimes remained colourless, suggesting either that there is little magnesium in the surface layers of the granules or that it is in some form which does not absorb the dye. Larvae fed on magnesium-enriched media gave similar reactions, except that the cells of the granule region of the tubules stained bright red and the granules pink. This staining was not confined to any particular type of granule. Solution of the granules in dilute acid, precipitation of magnesium as the hydroxide following addition of alkali, and addition of Titan yellow, produced a light pink precipitate from control larvae and a distinct red precipitate from magnesium-fed larvae.

Magneson II gave a rather more general coloration of the body than Titan yellow. The characteristic blue magnesium coloration was, however, most intense in the tubules and particularly in the granule-accumulating regions. Granules from control larvae stained a light blue, and those from larvae on magnesium-enriched media a darker blue. All granules stained similarly. Of the remaining portions of the body the hindgut stained more intensely than the midgut.

There is no doubt, therefore, that the malpighian tubules, and particularly the granule-accumulating regions, are richer in stainable magnesium than the other organs and tissues.

(j) Barium and Strontium

In third instar larvae fed on control medium, red staining after treatment with sodium rhodizonate (Waterhouse, unpublished data) was distinct in the granule-accumulating regions, and faint elsewhere in the tubules. Staining was most intense towards the posterior blind ends of the granule-accumulating regions and somewhat less intense near the bends. The intervening portion of the tubule, although stuffed with granules, either stained very lightly or not at all. Larvae fed on liver, brain, or muscle reacted similarly except for the occasional larva feeding on dehydrated mutton, which accumulated no granules (see Section VI(g)). These larvae did not stain at all.

Granules from a number of control larvae were mounted in sodium rhodizonate solution. A high proportion from some larvae were stained but, more frequently, only a very small proportion reacted. Sometimes no stained granules could be seen. All sizes and types of granules were stained at one time or another, although apparently identical granules from the same larvae remained unstained. The colour of the stained granules varied from pink to

dark red, being sometimes uniform throughout the granules, at others appearing only in the outer layers. There is strong evidence, therefore, that either barium or strontium, or both, are present at times in the granules. Staining occurred in a somewhat similar fashion in young second instar larvae, but the intensity was less.

The malpighian tubules of larvae fed on barium-enriched medium (0.5 ml. 1 per cent. barium chloride per 10 ml. medium) stained even more intensely than control larvae. All types of granules stained, but those which had an unhomogeneous interior composition reacted most frequently. Injection into the haemolymph of a dilute solution of barium chloride in saline, followed by rhodizonate treatment, resulted in fairly generalized light staining of the body with the granule region of the tubules almost always heavily stained and certain midgut cells sometimes distinctly stained. When barium is readily available, therefore, it is accumulated both in the granules and in the cells of the granular region in amounts greater than in control larvae.

The yellow portions of the malpighian tubules were seldom as intensely stained as the granule-containing regions. On several occasions barium-fed larvae had small deposits of granules in the lumen of the yellow tubules near their blind ends. These granules and the surrounding tubule cells stained more intensely with rhodizonate than the remainder of the yellow tubules. In pupae, as in larvae, staining occurred in the granule-accumulating portions of the tubules.

The malpighian tubules of larvae fed on strontium-enriched medium (0.5 ml. of 2.5 per cent. strontium chloride per 10 ml. medium) stained in a very similar fashion to those of larvae fed on barium-enriched medium, although the colour intensity was somewhat less, particularly in the yellow regions of the tubules. Nevertheless, it was more intense than encountered in control larvae. Examination of granules mounted in sodium rhodizonate solution showed that fewer were stained than with barium-fed larvae, but generally more than with controls. Most of the stained granules were medium-sized and there was often a good deal of intensely staining non-granular debris present. It appears, therefore, that strontium, too, can enter into the formation of the granules.

Definite evidence of the presence of both barium and strontium in control larvae was obtained by pretreatment with chromate (Waterhouse, unpublished data). Owing to the formation of unreactive barium chromate, this treatment prevented the characteristic intense coloration in tubules of larvae fed on barium-enriched medium. It did not interfere with tubule coloration of larvae on strontium-enriched medium, strontium chromate being capable of reacting with the rhodizonate solution. When this treatment was applied to control larvae or larvae fed on liver, some red coloration was usually observed in the granule-accumulating region, although this was not as intense as usual.

Finally, both barium and strontium were detected spectrographically in small amounts in the granules from control larvae, the former in a concentration between 0.005 and 0.001 per cent. dry weight, and the latter between 0.02 and 0.01 per cent. dry weight.

(k) Iron and Copper

The absence of stainable iron from the tubules, even when larvae have been fed on iron-enriched media, has already been described (Waterhouse 1940*b*). It is interesting to note, however, that, following the injection into the haemolymph of small amounts of saline saturated with ferric ammonium sulphate, the only portions of the body staining for iron by the Prussian blue reaction are the middle region of the midgut and the granule-accumulating portions of the tubules. In the latter, staining is generally most intense at the anterior and posterior ends and less intense towards the centre. Under these conditions stainable iron is not taken up by the yellow regions of the tubules.

Copper can generally be detected in the tubules of control larvae by the diethyldithiocarbamate reaction. It is present in the yellow portions, but also occurs at times in the granule-accumulating regions (Waterhouse 1945*a*). The ability of the cells of the granule region to take up copper from the blood was confirmed by injection into larvae of a dilute solution of copper sulphate in saline. The middle region of the midgut also stained more intensely than usual.

(l) Sodium and Potassium

Traces of sodium were detected in the granules by the zinc uranyl acetate test, but potassium was not detected either by sodium cobaltinitrite in acetic acid or by dipicrylamine (Feigl 1947).

(m) Ammonia

Solutions used for the detection of ammonia were Nessler's and Riegler's reagents (Feigl 1937, 1947). Although Nessler's reagent failed to produce any coloration of the tubules, Riegler's reagent unmistakably indicated the presence of ammonia therein. Thus a distinct red coloration developed in the cells of the tubules with local variations here and there in the depth of colour. Addition of saturated calcium hydroxide to render the solution slightly alkaline intensified the reaction and also prevented solution of the granules in the reagent. Many granules were stained light to dark yellowish-brown. This staining was not confined to any particular type of granule, but was more distinct in granules taken from stained tubules than in granules removed from the tubules and stained separately. It is clear, therefore, that ammonia either enters into the formation of the granules or, at least, is present in the fluid surrounding them and thus may be absorbed on to the granules during testing to produce staining. This agrees with the results of Lennox (1941), who showed quantitatively that more ammonia was present in the tubules than anywhere in the body except the hindgut. Unfortunately no distinction was drawn between the granule-accumulating portion and the remaining portion of the tubules. Furthermore, his analyses were based on aqueous extracts of organs, so that ammonia bound in the water-insoluble granules would not have been recorded.

(n) Uric Acid

Two principal tests were employed for the detection of uric acid. One was the intense blue colour produced by arsenophosphotungstic acid and sodium cyanide (Benedict 1922), the second employed the modified reagents of Newton (1937). Unfortunately neither of these methods is completely specific. Several other naturally occurring reducing agents produce a blue coloration which is, however, generally much less intense than that produced by uric acid. Thus Brown (1938a) found that cysteine and tyrosine gave faint colour reactions and Blaich and Koch (1937) added glutathione, ascorbic acid, cystine, glucose, and resorcinol to the list of reactive materials. Newton's reagents do not react with phenols (e.g. tyrosine, resorcinol), but the remaining materials presumably still interfere if present in sufficiently high concentration in the tissues.

Since the uric acid reagents react with nickel-coated pins, fine glass rods were used for holding down the larvae. Several minutes were required for the colour to reach full intensity and it faded again after standing for some time. With Benedict's reagents the blue coloration characteristic of uric acid appeared in the cut edges of the cuticle, in the skeletal muscles, and rather irregularly along the yellow portions of both anterior and posterior pairs of malpighian tubules. It occurred infrequently in the midgut, but more frequently in the hindgut, particularly just posterior to the entrance of the tubules. The hindgut contents were frequently stained a pale blue. A transient pale blue coloration was sometimes observed in the cells of granule-accumulating regions of the tubules, but more frequently no coloration occurred, even in larvae fed from hatching on media containing 5 per cent. uric acid. No coloration of the granules was ever observed. The cells of the granule-accumulating regions, in common with most other previously unstained tissues, did stain a light blue after injection of a saturated solution of uric acid in saline.

To determine whether the absence of staining was, perhaps, due to poor access of the reagents the granule-accumulating region was dissected out, dried, defatted with acetone, dried again, and the reagents added. The granules still failed to stain, although a similar treatment of previously unreactive fat body produced an intense blue coloration. When granule-containing portions of the tubules were treated with dilute hydrochloric acid and the reagents added, no more than a light blue colour ever developed, indicating that only slight traces of uric acid or urates could be present. This finding was confirmed with Newton's reagents (Newton 1937) which produced a far more intense coloration of larvae than Benedict's reagents, but no coloration of the granules or of a solution obtained by dissolving these in a sodium tungstate-sulphuric acid mixture.

Three confirmatory tests were performed. The reagent 2,6-dichloroquinone chloroimide (Fearon 1944) gave a golden-brown coloration in the same tissues which reacted above. The granules did not stain. Application of 0.25N silver nitrate produced blackening of cells and darkening of granules in the accumulating region, both changes being resistant to ammonia treatment;

on the other hand, the well-known Murexide test for uric acid gave a negative result. Both of these tests are regarded with suspicion by Lison (1936). Brown (1938a) found on analysis that, although uric acid was present in *L. sericata* larvae and that, although Benedict's method indicated its presence in the excreta, uric acid was, in fact, not excreted as such, being first oxidized to allantoin. He suggested that an interfering substance in his analyses was probably tyrosine, produced from the casein of his larval medium. To sum up then, the absence of distinctive coloration from the accumulating region means that, if uric acid or urates are constituents of the granules, they must be present only in extremely small quantities.

In spite of the caution which must be observed in interpretation of results, some confidence was gained in the Benedict colour reaction by its application to pupae and to adults. Thus, in the late pupal stage, a very intense blue coloration was given by the fat body, the meconium, and both the yellow and the granule-accumulating regions of the malpighian tubules. Earlier, in studying events towards the end of pupal life, the appearance of small granules in both anterior and posterior pairs of tubules was described. The newly formed granules from the posterior pair were first tested for uric acid, being, to all appearances, identical with the newly formed granules of the anterior pair and uncontaminated, as are the latter, by larval granules. The newly formed granules differed markedly from the larval granules not only in appearance, but also by being sparingly soluble in 1N hydrochloric acid without evolution of gas, and by being soluble in 5 per cent. sodium cyanide. Furthermore, the cyanide solution of granules gave a very intense uric acid reaction, so that there is no doubt that they contain large amounts of uric acid or urates. Like the larval granules, however, they contain no protein (see (o)). Additional tests demonstrated that, late in pupal development, uric acid or urate granules are present in the granule-accumulating as well as in the yellow regions of the anterior pair of tubules. The larval granules, awaiting discharge from the granule-accumulating region, still do not react. Urate granules are also present in the adult, being predominantly in the yellow regions of the tubules and in the hindgut. Brown (1938a) has shown that there is a rapid rise in uric acid content during pupal development and a sharp fall on emergence, due to the excretion of uric acid in the meconium. Considerable quantities of uric acid are also produced and excreted during adult life. This coincides with the picture obtained histochemically.

(o) Protein

To determine whether protein entered into the formation of the granules as a nucleus for the deposition of salts, one large mass of granules was dissolved in 10 per cent. trichloroacetic acid and another in 1N acetic acid. After allowing the slides to dry, ninhydrin in 50 per cent. glycerine was added and the slides heated. Although a faint blue coloration was seen at the edges of the dried deposit produced by acetic acid treatment, no staining of particles was observed. One must conclude, therefore, that protein is absent. In this

respect, the granules differ from the calcospherites examined by Keilin (1921) in which an albuminoid stroma of calcoglobulin remained after acetic acid treatment. Small particles of organic material also remained after solution of the granules from the tubules of *Psychoda* larvae (Krüper 1930).

(p) *Oxalate*

In view of the many records of the presence of oxalic acid in excretory granules (Uvarov 1928; Wigglesworth 1939), mostly based upon the doubtful recognition of crystal structure, the sensitive aniline blue test (Feigl 1947) was performed. The tests were uniformly negative even when granules were examined which had been obtained from larvae growing on medium containing 0.5 per cent. oxalic acid.

(q) *Lactate*

Lactic acid could not be detected in the granules by the tests involving the production of characteristic colours with *o*- or *p*-hydroxydiphenyl and sulphuric acid.

(r) *Sulphydryl*

The recently described red stain (1 [4 chlormercuric phenyl azo] naphthol 2° (Bennett 1948*a*, 1948*b*), which has a high specificity for sulphydryl groups) was used as a saturated solution in *n*-propyl alcohol. Most of the tissues and organs, including the yellow regions of the tubules, were heavily stained. Other tissues appeared only lightly stained, such as the tracheae and the cells of the granule-containing portion of the tubules, whilst the peritrophic membrane and cuticle (but not the underlying hypodermal cells) remained unstained. The granules did not stain. From these reactions it is clear that free sulphydryl groups do not occur in particularly high concentration in the granule-accumulating region of the tubules.

VI. EFFECT ON GRANULE FORMATION OF ALTERATIONS TO DIET

(a) *Calcium*

The calcium content of the medium was increased by the addition of various soluble salts. A six-fold† addition of calcium caused an enlargement in diameter of the granule-accumulating region and its extension around the bend into the upper portion of the descending arms of the tubules. Deposits of granules increased further with increasing calcium concentration, adverse effects (growth retardation) first becoming noticeable with a 36-fold addition. These retarded larvae accumulated massive deposits of granules which occupied the upper half to two-thirds of the descending arms, in addition to the ascending arms of the tubules. A small accumulation of granules in the blind ends of the posterior pair of tubules was also a common feature. It was estimated that the amount of granular material which fully grown larvae, fed

* A sample of this reagent was supplied through the courtesy of Dr. H. S. Bennett.

† An *x*-fold addition means that *x* times as much of the element as was already present in the control medium was added.

on the 36-fold addition, had accumulated was about eight times that accumulated on control medium. It is a general feature that, as the calcium concentration in the medium increases, the ascending arms of the anterior tubules do not run straight back to the posterior end of the body as in control larvae, but are convoluted and this, with increased tubule diameter, provides the additional storage space. Granules having diameters up to $20\ \mu$ were not uncommon and there were many in the 10 to $15\ \mu$ range. The former are larger than are found in control larvae.

Adults fed a saturated solution of calcium gluconate accumulated many more granules of a larger average size than adults fed sugar solution. More granules were present in the blind ends of the anterior than of the posterior pair of tubules.

(b) *Magnesium*

A two-fold addition of magnesium as glycerophosphate resulted in a slight increase in the number of granules accumulated. Both a 12-fold addition of magnesium, which resulted in only very slight growth retardation, and a 20-fold addition, which caused severe retardation, produced large quantities of granules. A feature of similarity to larvae fed on calcium-enriched media was that the ascending arms of the tubules had several bends in them, thereby considerably increasing their length. However, on a magnesium-rich diet, not only was the region of the tubules near the blind end seldom enlarged in diameter, but there was a tendency for the distended portion of the descending arms to be filled with fluid and for relatively few granules to be present. The same situation was also commonly seen in the posterior third of the granule-accumulating region. This distension of the tubules with fluid appears to be characteristic of sublethal concentrations of magnesium in the food. As with calcium, there were small numbers of granules at times in the blind ends of the posterior pair of tubules.

Adults fed sugar solution containing magnesium glycerophosphate accumulated far more granules than those fed solution alone. These granules occurred in larger numbers in the anterior pair of tubules. Feeding either magnesium or calcium-rich foods, therefore, produces granule formation in adults, as in larvae, and the frequent absence of granules in adults is largely due to the low concentrations of these elements in their diet.

(c) *Phosphate*

The first distinct effect was with a three-fold addition of phosphate, when noticeably less granular material was formed. With an eight-fold addition, the overall picture was that of a considerable reduction in tubule diameter (granule-accumulating region only), while the deposits were markedly smaller than in control larvae. In particular, very few granules were present in the blind ends of the tubules. In some larvae, short regions of the tubules were relatively transparent, due to the presence in the lumen of fluid carrying few granules, a condition somewhat reminiscent of the far more extensive transparency associated with additions of magnesium. Larval growth was normal at this concentration of phosphate.

Adults fed sugar solution containing 2.5 per cent. potassium dihydrogen phosphate accumulated no granules.

(d) Mixtures of Calcium, Magnesium, and Phosphate

Larvae grown on media containing two-fold additions of both calcium and phosphate were indistinguishable from control larvae in amount of granular deposit. Since a two-fold addition of calcium alone produced an increase in granular material it appears that the increased phosphate partially inhibited granule formation. Similarly, although a two-fold addition of calcium and magnesium produced a slightly greater accumulation of granules than a two-fold addition of either calcium or magnesium alone, a two-fold addition of calcium, magnesium, and phosphate produced about the same amount of granular material as with calcium alone.

On the other hand, when a three-fold addition of calcium and magnesium and a 1.5-fold addition of phosphate was made, the tubules were far better filled than those of control larvae. It is clear that there is a rather complicated interrelation between the identity and both relative and absolute concentrations of various substances in the diet in their effect on granule accumulation. This is clarified in the discussion.

(e) Barium or Strontium

The addition of barium or strontium to the medium in concentration sufficient to produce growth retardation appeared to increase slightly the amount of granules accumulated. Both metals are toxic in concentrations (about 0.002 per cent. and 0.003 per cent. dry weight respectively) very much lower than the concentrations of calcium (0.04 per cent.) and magnesium (0.12 per cent.) normally present in the control medium. The amounts of barium and strontium available to the larvae would not, therefore, be expected to produce greatly increased numbers of granules unless these metals were accumulated preferentially.

(f) Carbonate or Bicarbonate

The addition of sodium carbonate or bicarbonate to the medium had no effect on granule accumulation. The carbonate apparently did not prevent any appreciable amounts of the calcium and magnesium of the medium being absorbed from the alimentary canal. However, with carbon dioxide already available in the larval tissues in excess over needs for granule formation (see Section IX), additional carbonate or bicarbonate would scarcely be expected to produce any effect.

(g) Meat-fed Larvae

Larvae fed on sheep's liver, brain, or muscle accumulate relatively small quantities of granules. Occasional larvae fed on reconstituted dehydrated mutton accumulated none at all. Poor accumulation of granules is no doubt due to the lower concentrations of calcium and magnesium in these diets.

(h) Citrate

Citrate has frequently been used as a decalcifying agent. However, concentrations of citric acid and sodium citrate which caused some growth retardation of larvae depressed granule formation only slightly, if at all.

(i) Oxalate

Oxalic acid, incorporated in the medium in a concentration sufficient to produce slight growth retardation, had no apparent effect upon granule accumulation. It was thought that the formation of relatively insoluble calcium oxalate in the food might depress granule formation by lowering the amount of calcium available.

(j) Phloridzin

Phloridzin had no effect upon granule formation. Gutman, Warwick, and Gutman (1941) reported that phloridzin prevented calcification of cartilage by inhibiting a phosphorylative glycogenolytic enzyme system, but this system is evidently unimportant in granule formation.

(k) Sulphanilamide

Sulphanilamide, in concentrations sufficient to cause some growth retardation, had no effect on granule formation. The granules formed dissolved in dilute acid with the evolution of carbon dioxide in the same way as control granules. The effect of feeding sulphanilamide was investigated because this material has been shown to inhibit carbonic anhydrase, which catalyses the production of carbonate at sites of calcification in vertebrates (Mann and Keilin 1940). Since sulphanilamide had no effect on granule formation, either carbonic anhydrase is not important in this process or the sulphanilamide did not reach the granule-accumulating regions. The former is supported by the failure to detect carbonic anhydrase by the method of Philpot and Philpot (1936) either in ground-up larvae or in extracts of the malpighian tubules.

VII. THE pH AND EH OF THE TUBULES

(a) pH Indicators

Saturated solutions of indicators were injected into larvae or added to dissections (Table 1).

The contents of the yellow regions of the tubules have a pH of 7.4 to 7.8, the pH often varying slightly, but irregularly, along the length of the tubules. The cells have a similar or slightly lower pH, as can sometimes be seen after prolonged irrigation with indicator solutions. Indicators are taken up by the granule-accumulating region only after lengthy contact and then principally in the portion immediately adjoining a yellow region. It is possible that much of the indicator has diffused in from these yellow regions. In all cases the colour of the indicator was the same in the accumulating regions as in the yellow regions or slightly more alkaline.

A feature of these tests was the rapid uptake of indicator by the midgut, the various regions of which exhibited pH values already noted (Waterhouse 1940*a*). The midgut caeca, which did not stain when indicator media were fed, stained following injection and had the same pH as the anterior midgut. Indicators were also absorbed by the isolated hindgut, being visible in the lumen and in some, but not all, of the hindgut cells.

The adult tubules have a slightly lower pH than those of larvae, namely 7.2 to 7.6. Occasionally some regions, which varied from adult to adult, but which generally occurred towards the outlet into the gut, had a pH lower than the bulk of the tubules. The reason for these apparently more acid regions was not determined, but they may have been due to the presence of urates. This is suggested by the acid pH of the meconium (6.2 to 6.5), urates (presumably acid) having been added to the slightly alkaline larval granules to form the bulk of the solid material in the meconium. The portions of the adult tubules corresponding to the larval granule-accumulating regions did not take up indicators nearly as readily as the remainder of the tubules but, when indicator was visible, values similar to the remainder of the tubules were obtained.

TABLE 1
pH OF MALPIGHIAN TUBULES AND BLOOD

Indicator	Larvae 2.5 Days Old			Adults Malpighian Tubules
	Malpighian Tubules		Blood	
	Yellow Region	Granule Region		
Brom-cresol purple	> 6.4	> 6.4	> 6.4	> 6.4
Phenol red	7.4-7.8	7.4-7.8	7.2-7.6	7.2-7.6
Meta-cresol purple	About 7.8	About 7.8	< 7.8	< 7.8
Cresol red	< 7.8	About 7.8	< 7.8	< 7.8
Range	7.4-7.8	7.4-7.8	7.2-7.6	7.2-7.6

(b) *Eh Indicators*

One per cent. solutions in saline (or saturated solutions if the solubility was lower) of the indicators shown in Table 2 were injected into larvae. These were generally taken up rapidly by the tubules. After large doses, indicators were also often taken up by the gut cells and fat body. The addition of hydro-sulphite or peroxide was sometimes useful in deciding whether the indicator was in the oxidized or reduced state.

Taking pH 7.6 as the mean value for the larval tubules, an *Eh* in the range 0.13 volts to 0.16 volts is indicated. The granule-containing regions of the tubules were never as intensely coloured as the yellow regions owing, presumably, to a slower dye uptake, but no difference in the character of the coloration could be observed. In spite of the rather poorer supply of tracheae to the granule-accumulating regions, oxidizing conditions are apparently similar to those in the yellow regions. No confirmation was therefore obtained

TABLE 3

ANALYSES OF LARVAL TISSUES FROM SIX BATCHES OF LARVAE GROWN ON THE STANDARD ARTIFICIAL MEDIUM, AT 30°C.
CULTURES 1 TO 6

[illegible]

* Average of 2 analyses.

† Average of 3 analyses.

† Average of 4 analyses.

§ Taken from Waterhouse (1945*b*).

of the indication from riboflavin uptake that the Eh was higher in the granule region than elsewhere in the tubules. The potential of the blood is 0.14 to 0.17 volts. This value is close to that of 0.12 volts determined with indicators for *Sarcophaga* larva by Dennell (1947).

VIII. CHEMICAL ANALYSES

Analyses were carried out on the granule-containing portion of the malpighian tubules (referred to as "M.T.—granules" in the tables), as well as on various other larval tissues for comparison. The yellow portions of both pairs of tubules were pooled for analysis. Table 3 gives individual analyses for calcium, magnesium, and phosphorus for larvae from six different cultures. Cultures 1 to 4 were dissected when larvae were 2.5 days old, culture 5 at 3.5 days, and culture 6 at 4.5 days. As there was no apparent difference between these age groups the results were averaged.

TABLE 2
EH OF TUBULES AND BLOOD OF *L. CUPRINA* LARVAE 2.5 DAYS OLD

Indicator	Eh (volts)	
	Tubules (pH 7.6)	Blood (pH 7.4)
Thionine	> 0.043	> 0.050
1-Naphthol 2-sodium sulphonate indo 2,6-dichlorphenol	> 0.073	> 0.088
1-Naphthol 2-sulphonate indophenol	> 0.087	> 0.099
Toluylene blue	> 0.094	> 0.101
Sodium 2,6-dibromobenzenone 3-methoxyindophenol	(> 0.130*)	(> 0.140*)
<i>o</i> -Cresol indophenol	< 0.156	< 0.168
<i>m</i> -Cresol indophenol	< 0.173	< 0.185
Phenol indophenol	< 0.186	< 0.200
<i>o</i> -Brom-phenol indophenol	< 0.184	< 0.200
Range	0.13 to 0.16	0.14 to 0.17

* All values except these taken from Hewitt (1936); these approximate values were arrived at by analogy with pH curves for the other indicators.

An important factor, leading to variation in analyses of granules, is their contamination by constituents from the encircling cells. These form only a small proportion of the total weight when the tubules are well filled with granules, but a far higher proportion when the tubules are less well filled. It follows that granules of identical composition will give different percentage values depending on the ratio of weight of granules to weight of cell constituents. This factor, however, does not account for the entire variation found.

The phosphorus, calcium, and magnesium contents of the granular region of the tubules is, on the average, 20 to 30 times higher than that of other

portions of the larva. Indeed, although comprising only 0.9 per cent. of the dry weight of the larva, the granule region contains 28 per cent. of the inorganic phosphorus, 45 per cent. of the calcium, and 36 per cent. of the magnesium. There is, therefore, no room for doubt that the granules are rich in these elements. It should also be noted that, on the average, they contain slightly more magnesium than calcium. The relatively high content of the yellow region of the tubules and the invariably higher content of the hindgut than the midgut for these three elements is readily explained by the assumption that these elements are continually being absorbed from the haemolymph by the malpighian tubules and that, while portion is retained in granular form, the remainder is excreted. Selective removal of other constituents from the food in the midgut might account for higher concentrations of these materials in the hindgut, but not in the yellow regions of the tubules.

TABLE 4
TOTAL AND INORGANIC PHOSPHORUS CONTENTS OF TISSUES OF *L. CUPRINA* LARVAE
CULTURES 7 TO 10

	7		8		9		10	
	Total	Inor- ganic	Total	Inor- ganic	Total	Inor- ganic	Total	Inor- ganic
M.T.—granules	13.4	12.6	17.0	11.9				
M.T.—yellow regions	1.45	0.61	1.48	0.33				
Hindgut	1.62	0.54	1.56	0.68				
Midgut	1.08	0.20	1.69	0.37				
Cuticle + muscles	1.06	0.29	1.14*	0.26*				
Fat body	0.99	0.13	1.11	0.06				
Blood	0.99	0.35	1.11	0.30				
Whole larva	1.22*	0.48°	1.10†	0.44†	1.41**		1.29‡	0.33§
Av. larval wt. (mg.)	28.6		33.4		31.5		14.2	
Moisture (%)	79.5		76.1		77.5		79.6	

* Average of 2 analyses.

** Average of 3 analyses.

† Average of 4 analyses.

‡ Average of 6 analyses.

§ Average of 16 analyses.

The accumulation of phosphate in the granules was further demonstrated by feeding larvae on medium containing radio-active P^{32} . The granule-accumulating region of the tubules gave a count which was at least 15 times that of the yellow region and which also indicated a far higher phosphorus concentration than in the remaining tissues. A comparison was made (Table 4) of the total and inorganic phosphorus contents of the various tissues.

While it is clear that the granular region of the tubules contains some organically bound phosphorus the amount present is far less than the amount of inorganic phosphorus, and probably comes from the cells surrounding the granules. On the other hand, in the remaining organs and tissues, organically bound phosphorus is present in far greater amount than inorganic phosphorus.

By contrast, figures for total calcium and magnesium were no higher than those for inorganic calcium and magnesium, indicating that only very small amounts of these elements can be present in organic combination.

Table 5 gives results of analyses of the granule-containing portion of the malpighian tubules for phosphorus, calcium, magnesium, and carbonate. On the average, 3 per cent. of the dry weight of the tubules was due to carbonate. In the course of these experiments it was found that all of the gas evolved was absorbed by potassium hydroxide and hence was carbon dioxide.

TABLE 5
ANALYSIS OF THE GRANULE-ACCUMULATING REGION OF LARVAL MALPIGHIAN TUBULES
CULTURES 4 TO 6 AND 11

	% Dry Wt.				
	11	4	5	6	Av.
P	14.7	13.6	12.3	11.1	12.9
Ca	7.1*	6.2	7.2	7.4*	7.0
Mg	8.6*	11.3	9.2	10.0*	9.8
CO ₃	1.6*	5.1	3.3	2.4	3.1
Residue from P analysis	12		19	22	16
Residue from Ca and Mg analyses	7	15	25	14	
Av. larval wt. (mg.)	28.6	21.1	27.8	36.1	
Moisture (%)		83.1	80.1	79.5	
Age (days)	2.5	2.5	3.5	4.5	

* Average of 2 analyses.

To obtain some information on the amount of insoluble residue, which has been referred to earlier as a source of variation when calculating percentages for the granules, the coverslips carrying the samples were dried and reweighed after treatment with trichloroacetic acid, giving figures which varied from 7 to 25 per cent. The vigorous evolution of carbon dioxide following acid treatment could be seen at times to result in fragments of tubule being dislodged from the coverslip. More reliance should, therefore, be placed on the higher than on the lower values.

TABLE 6
ANALYSES OF THE ARTIFICIAL MEDIUM

% Dry Wt.			
P		Ca	Mg
Total	Inorganic	Inorganic	Inorganic
0.68*	0.40†	0.04‡	0.12‡
Moisture content 77%			

* Average of 3 analyses.

† Average of 4 analyses.

‡ Average of 8 analyses.

Analyses of the medium (Table 6) showed that it was about as rich in inorganic phosphorus as whole larvae, but contained less organic phosphorus, calcium, and magnesium.

In Table 7, typical analyses are shown of the granule-containing portion of the tubules from larvae grown on media to which various salts have been added. It should be noted that the figures for content per larva are not exactly proportional to those for per cent. dry weight. This is due to the variation from larva to larva in the weight of granules accumulated and the fact that tubules from different larvae were used for the various analyses.

TABLE 7
THE EFFECT ON THE COMPOSITION OF THE GRANULES OF ENRICHING THE
MEDIUM WITH SALTS

		Ca 24x	Ca 21x, P 1.5x	Mg 12x, P 4.5x	P 3x	P 6x	Av. for Control Larvae
% Dry wt.	P	7.1	6.9	13.7	12.2	12.0	11.0
	Ca	29.2*	20.0	3.2	11.0	6.3*	7.5
	Mg	1.7	0.7	14.6	10.8	7.4*	9.6
	CO ₃	21.8	18.1	4.3	4.7	8.9	3.1
γ per Larva	P	19.6	24.8	22.4	3.4	3.6	6.1
	Ca	106	67	4.6	3.4	2.1	4.0
	Mg	6.4	2.4	21.3	3.4	2.4	5.4
	CO ₃	85*	75.0	5.0	1.7	2.8	2.1
% Residue from	P	—	7.4	4.4	15	23.9	
	Ca and Mg	—	—	7.7	—	17.5	
Av. larval wt. (mg.)		29.1	28.5	25.4	26.3	23.1	
Moisture (%)		—	—	82.7	81.0	83.0	
Salt used		Calcium gluconate	Calcium glycero- phosphate	Magnesium glycero- phosphate	KH ₂ PO ₄	KH ₂ PO ₄	
Age (days)		3.5	5.5	2.5	2.5	2.5	

* Average of 2 analyses.

Addition of calcium to the diet clearly increased very greatly the amount of both calcium and carbonate in the tubules and this is almost certainly due to an increased amount of calcium carbonate. Although the percentage of magnesium present is far less than on control media, the actual amount present per larva falls within the range observed for larvae on control media (see Table 3), so that accumulation of magnesium has apparently not been interfered with. Whereas the percentage of phosphorus present is somewhat less than for control larvae, the actual amount per larva is considerably increased and one can therefore conclude that calcium phosphate is a constituent of the granules. Since no phosphate was added in the enrichment of medium with calcium gluconate it is clear that, in larvae on control media, phosphate is available in excess over requirements for granule formation, other factors (of which the concentration of calcium is one) limiting the amount of phosphate deposited.

Addition of magnesium increased considerably the amount accumulated per larva of magnesium and phosphate and, to a lesser extent, the amount of

carbonate. The amount of calcium accumulated was unchanged. Magnesium phosphate and magnesium carbonate are evidently constituents of these granules, which were also found to contain about 0.5 per cent. ammonia. If all of this ammonia is present, as might be expected, as the insoluble magnesium ammonium phosphate, this would bind only 5 per cent. of the magnesium present. These larvae each contained about 0.48 γ ammonia in granule form, which is considerably more than control or liver-fed larvae (about 0.068 γ) or calcium-fed larvae (about 0.088 γ). These latter figures correspond to an ammonia content in the granules falling within the range 0.1 to 0.3 per cent. It is clear, therefore, that only a small amount of the magnesium is present as magnesium ammonium phosphate.

Addition of phosphate had little effect upon the percentage composition of the granule region. This may be taken as confirmatory evidence that phosphate is available in excess in the control medium. The amounts accumulated per larva in the granule region of these larvae were equivalent to, or lower than, those at the lower ends of the ranges indicated in Table 3 for control larvae, general confirmation of the earlier observation that the addition of phosphate tends to inhibit granule formation.

Several analyses have been carried out on granules from larvae fed on sheep's liver (Table 8). Cultures *A* and *B* gave percentage values reminiscent of those on media rich in calcium (Table 7), in that both calcium and carbonate

TABLE 8
ANALYSIS OF GRANULE-ACCUMULATING REGION OF LARVAE FED SHEEP'S LIVER AT 27°C.

	Cultures	A	B	C	D	E
% Dry wt.	P	4.3	2.4	7.7*	6.9	6.5
	Ca	15.9*	15.7	6.7	0.5	3.5
	Mg	3.4*	5.1	5.7*	4.1	5.6
	CO ₃	16.3	15.4	—	1.6	0.8
γ per Larva	P	1.5	0.45	1.4*	1.1	0.8
	Ca	4.3*	1.6	1.3*	0.08	0.5
	Mg	0.8*	0.5	1.1*	0.6	0.8
	CO ₃	5.0	1.4	—	0.2	0.05
% Residue from	P	19.6	57.1	45.1*	31.2	14.0
	Ca and Mg	8.6	16.4	35.5	34.2	10.4
Av. larval wt. (mg.)		45.5	32.1	40.5	36.5	32.4
Moisture (%)		73.0	77.0	72.0	73.6	77.3
Age (days)		3.5	3.0	3.5	2.5	2.5

* Average of 2 analyses.

were abundant, while phosphorus and magnesium were present in relatively small amounts. On the other hand, culture *C* gave values not unlike those on the artificial medium, and cultures *D* and *E* indicated that, at times, more magnesium is present than calcium.

In all cases the weight of granules present per larva was considerably smaller than in larvae fed on the artificial medium. The extremely large variation in the relative amounts of material present is almost certainly due to the rather variable composition of liver (average for several analyses shown in Table 11). Further analyses of larvae fed on animal tissues were not performed since the main factors influencing the composition of the larval granules had been determined using the more uniform artificial medium. In addition, when only small quantities of granules are present, the mineral content of the cells of the tubules becomes an important interfering factor in the analyses.

IX. DISCUSSION

It has been established that the granules accumulated in the tubules of *L. cuprina* larvae contain calcium and magnesium phosphates and carbonates. It is highly probable also that part of the magnesium present occurs as magnesium ammonium phosphate. However, quite apart from the low values for ammonia found by analysis it is clear that most of the magnesium cannot be bound in this fashion because, if all of the magnesium were so bound, the granule region of control larvae, with an average of 9.4 per cent. magnesium, would contain about 95 per cent. magnesium ammonium phosphate, which would leave no place for the calcium salts. The relative amounts of calcium and magnesium in the granules varies with their relative concentrations in the food. When fed the standard artificial medium, which contains three times as much magnesium as calcium (Table 6), slightly more magnesium than calcium is present in the granules. A food containing equal weights of calcium and magnesium would therefore be expected to result in more calcium than magnesium being accumulated in the granules. However, since many animal tissues contain more magnesium than calcium (Table 11; Takamatsu 1936) calcium and magnesium are probably of about equal importance as normal granule constituents. Certainly the proportion of magnesium present is greater than has been imagined by earlier workers and contrasts strikingly with figures for bone in which 40 to 50 times more calcium is present than magnesium (Duckworth, Godden, and Warnock 1940). The contents of phosphorus, calcium, and magnesium in whole larvae and in larval blood recorded in the present analyses are of the same order of magnitude as those already recorded for other insects, including several Diptera (Table 9), so that there is nothing peculiar about *L. cuprina* larvae in this respect.

Specific staining reactions have failed to restrict any particular salt to any of the various granule types and it must therefore be concluded that all salts may occur in all types of granule. The appearance of certain granules suggests that they are built up of concentric layers of differing composition, although each layer may be either a homogeneous or heterogeneous mixture of salts. On certain highly enriched diets, however, it is possible that single-salt granules occur.

The presence of barium and strontium is perhaps surprising at first sight, particularly because of the toxicity of the former, although in fact it might quite reasonably have been expected. Any mechanism for the uptake of calcium and magnesium from the haemolymph would be expected to operate also for barium and strontium. Nevertheless, similar accumulations of barium and strontium do not appear to have been recorded in other animals.

TABLE 9
PHOSPHORUS, CALCIUM, AND MAGNESIUM CONTENTS OF DIPTERA

			g. per 100 g. Dry Tissue				
			P		Ca	Mg	Author
Species			Total	Inorganic			
<i>Sarcophaga</i>	<i>carnaria</i>	larvae on meat	0.87		0.46	0.23	Tomita and Kumon* 1936
<i>Calliphora</i>	<i>erythrocephala</i>	larvae on meat	0.76	0.39			Khouvine and Gre- goire* 1940
<i>Lucilia</i>	<i>sericata</i>	larvae-- on meat on blood	0.70-0.76 0.77				Hobson 1935
<i>Calliphora</i>	} adult thorax			0.39			Baldwin and Need- ham* 1933
<i>Lucilia</i>							
<i>Drosophila</i>		adults from larvae fed-- normal diet deficient diet			0.13 0.001		Rubinstein, Lwowa, & Burlakowa 1935
<i>Chironomus</i>	<i>tentans</i>	larvae	1.18		0.4	0.28	Birge and Juday* 1922
<i>Lucilia</i>	<i>cuprina</i>	larvae artifi- cial medium	1.2	0.36	0.14	0.24	Present analyses

* These figures were calculated from data provided by the respective authors.

It is worth considering whether measurements of the respiratory quotient of granule-accumulating dipterous larvae will give values affected by the calcium and magnesium contents of their food. According to Hitchcock and Haub (1941) 7.7 litres of carbon dioxide are produced per 100 g. *Phormia* larvae from the time they leave their food until adults emerge. At least as much must have been produced during larval development. Further, from the figures of Cousin (1932), one can calculate that large, feeding larvae of *L. sericata* produce an average of 440 ml. carbon dioxide per 100 g. larvae per hour, while small larvae respire at the rate of 660 ml. carbon dioxide per 100 g. per hour. In the present experiments, larvae fed on a diet enriched 24 times with calcium, which accumulated more carbonate-containing granules than on any other diet examined (see Table 10), bound in 3.5 days only a little over 100 ml. of carbon

dioxide in granule form per 100 g. larvae, which would certainly introduce much less than a 1 per cent. error in respiratory quotient determinations. On most diets, including animal tissues, the error involved would be still less than on this calcium-rich diet.

TABLE 10
AMOUNT OF CARBON DIOXIDE BOUND IN GRANULES BY LARVAE ON
DIFFERENT DIETS

Medium	Age of Larvae (days)	Av. CO ₂ /100 g. Larvae Wet Weight (ml.)
Control	2.5	3
Control	3.5	3
Control	4.5	3
Enriched P 3x	2.5	3
Enriched P 6x	2.5	5
Enriched { Mg 12x P 4.5x } }	2.5	8
Enriched { Ca 21x P 3x } }	5.5	111
Enriched Ca 24x	3.5	123

On the other hand, calliphorid larvae excrete large amounts of ammonia in close proximity to the most important respiratory openings, the posterior spiracles, which may seriously disturb a measurement of the respiratory quotient (Brown 1938*b*) by the formation of ammonium carbonate. It is possible, therefore, that even more carbon dioxide was produced than was recorded by Cousin (1932) and by Hitchcock and Haub (1941). Certainly, immersion of larvae in acids results in bubbles of carbon dioxide being produced from the anus and from the surface of the cuticle surrounding it. This is not due to the solution of granules, which are absent from these situations.

It is clear both from feeding experiments and from analyses that the quantity of granules accumulated and their composition depend upon the composition of the diet. On many of the diets more phosphate is present and, on all of the diets, more carbon dioxide is available within the larva than can be found in granule form; under these conditions the amounts of calcium and magnesium present limit granule formation. The smaller accumulations of granules in larvae fed on meat compared with those on the artificial medium would appear to reflect the somewhat lower concentration of calcium and magnesium and the equal or higher concentration of phosphorus in meat. Indeed, it appears probable that the low concentration in the larval food and the consequent small number of granules formed must have been responsible for the omission of Lowne (1890) and Pérez (1910) to mention the presence of granules in their extensive works on *Calliphora erythrocephala* larvae. Pérez did observe

the accumulation of granules in the pupal period, but these clearly correspond to the urate-rich granules formed in *L. cuprina* pupae.

Suitable figures for comparison with the artificial medium for the mineral content of sheep, cattle, and most other animal tissues and organs do not appear to be available, but what information can be calculated from the literature suggests that they are of the same general order of magnitude as the figures

TABLE 11
COMPARISON OF THE MINERAL CONTENTS OF MAMMALIAN TISSUES AND OF
THE ARTIFICIAL MEDIUM

	g./100 g. Dry Wt.		
	Ca	Mg	Total P
Human muscle [°]	0.024	0.079	0.76
Human blood [°]	0.034	0.025	0.28
Human brain [°]	0.05	0.066	2.3
Human liver [°]	0.024	0.044	0.46
Sheep blood [†]	—	—	0.125
Sheep blood [‡]	0.16	—	—
Sheep liver	0.02	0.07	1.58
Artificial medium	0.04	0.12	0.68

[°] Calculated from Shohl 1939.

[†] Calculated from Hobson 1935.

[‡] Calculated from Ray 1939.

for the human body (Table 11). Human muscle, for instance, contains about half as much calcium and magnesium as the medium. Since addition of calcium or magnesium salts to sheep or beef muscle considerably increases the amount of granular deposit, it can only be in having too low a concentration of these minerals that meat is unsuitable for granule accumulation. The different amounts of both calcium and magnesium available in the food for the two groups of species may possibly explain the accumulation of calcospherites in the fat body of leaf-mining Agromyzidae (Henneguy 1897; Keilin 1921), but not in predatory species (Thorpe 1930), and this may also hold for the reverse situation existing in the Limnobiinae (Tipulidae) where larvae of some predaceous species contain granules in their malpighian tubules, while plant-eating species do not (Könnemann 1924).

Nor is it particularly surprising, as has been mentioned in the literature, that the larvae of *Auchmeromyia* (Calliphoridae), which feed only on the blood of mammals, should accumulate granules, since calcium is present in blood in higher concentration on a dry weight basis than in the artificial medium. Admittedly, magnesium is present in about a quarter the concentration and phosphorus in less than half the concentration present in the artificial medium. However, even if phosphorus is not present in excess of metabolic requirements, and Hobson (1935) states that growth of *L. sericata* larvae on blood is improved by the addition of phosphate, there must be all the carbon dioxide required to fix any calcium available for granule formation. Furthermore, it has been found

that, in *L. cuprina* larvae, granule formation is partly inhibited by high concentrations of phosphate in the diet, although the larvae are otherwise normal. This effect is probably due to the retention of much of the calcium and magnesium in the alimentary canal as insoluble phosphates, and is overcome by enriching the medium with calcium or magnesium.

In addition to an adequate supply of the materials needed for granule formation, there must also be a specialized region of the malpighian tubules where granules can form and from which they are not flushed continuously into the hindgut. There are, for example, many blood-sucking insects which do not accumulate granules. In *L. cuprina* and in other calliphorid larvae the accumulating regions can easily be distinguished in general appearance from the remainder of the tubules, even when portions within the region contain no granules. The epithelium in the granule region of the larval tubules appears to be specially adapted to secrete into the lumen a number of materials including magnesium, calcium, and other alkaline earth metals as well as phosphates and bicarbonates or carbonates. Urates and several dyes, which are principally taken up by the yellow regions of the tubules, are not absorbed. While aqueous solutions are probably necessary for the transportation of the materials into the lumen, water must be rapidly reabsorbed or the granules would tend to be flushed out.

An analogous secretion of certain dyestuffs into the lumen of the tubules, followed by reabsorption by the cells which secreted them, has been noted in *Forficula*, *Periplaneta*, and *Dermestes* by Lison (1942). Certainly in *L. cuprina* a marked difference exists in the rate at which water is discharged into the lumen of the two regions. Thus, when tubules are placed in hypotonic saline, water uptake forces the granules further into the blind ends of the tubules and not in the direction of discharge. From this it might be thought that the granule regions are merely blind ends of the tubules into which the yellow regions direct excretory products for storage. This is not so since, under normal conditions, no packing of the granules into the blind ends can be observed. In addition, granules already in the blind ends of the tubules increase in size with increasing age of the larva, so that additional layers must be added *in situ* in spite of an intervening length of tubule packed with granules. Furthermore, following injection, iron is taken up by the granule-accumulating regions and not by the yellow regions.

In larvae fed on diets enriched with suitable calcium and magnesium salts a functional modification of the tubules, dependent upon the composition of the food, appears to have taken place. Granules are accumulated in the descending arms of the anterior tubules, a location in which they are not found in meat-fed larvae. These granules are not excreted as soon as they are formed as would be expected if the cells of this region retained their usual capacity to discharge materials rapidly into the lumen for transport to the hindgut. Instead, they are retained and increase in size as in the true accumulating regions. Experiments with dyes indicate that the permeability of

the granule-containing "yellow" regions has diminished to that of the true granule-accumulating regions. Furthermore, following injection, iron is now taken up by these cells which hitherto failed to absorb it.

Within the single pair of tubules involved in granule accumulation a different location of granules has been noted in different groups of dipterous larvae; for example, the entire tubule in *Stratiomyia* (Vaney 1900), the ascending tube in *Lucilia*, the distal part only of the ascending tube in *Thrixion* (Pantel 1898), and in the dilated middle region in *Ptychoptera* (Pantel 1914). This varied distribution is probably closely associated with the occurrence of a specialized tubule epithelium, but is also dependent upon the composition of the food, since it has been shown in *Lucilia* that granules may either be almost completely absent (on some meat diets), or present in the anterior half of the descending tubule as well as in the ascending arms after feeding on calcium- and magnesium-rich diets.

In adults there is less differentiation of the tubules although, in *L. cuprina*, the region corresponding to the larval granule-accumulating portion can still be distinguished from the rest by the smaller amount of yellow pigment present. Accumulations in adult flies have been recorded only in *Drosophila* (Eastham 1925), which were feeding on the same food as the larvae. Certainly, granules were absent in wild *Drosophila* adults caught in Canberra. In adult *L. cuprina*, granules are normally absent, but may be present if the diet contains quantities of calcium and magnesium salts. When present, they are more numerous in the relatively unpigmented region of the tubules than elsewhere, indicating that these regions are still specially adapted for granule formation. The discharge of granules from adult tubules after transfer from a mineral-rich to a mineral-poor diet indicates that two factors are responsible for the normal absence of accumulations in the adult. The first is the inadequate amount of calcium and magnesium salts available from the diet and the second is that, as granules are formed, they are continually being discharged from the tubules.

There is no support for the statement by Stewart (1934) that *L. sericata* larvae exude calcium carbonate through their body wall and, indeed, Stewart himself gives no adequate evidence that this may occur. Roubaud (1913) states that granules are excreted continuously by *Auchmeromyia* larvae and this may be true. In *L. cuprina* on diets which produce very large accumulations, causing invasion of granules into the yellow regions of the tubules, some are excreted but, if this does occur in larvae fed meat or the artificial medium, it is certainly unusual. There is no reason to doubt, however, that some calcium is normally excreted, although not in granule form. Neither the larval cuticle nor the empty pupal cases effervesce when immersed in acid as they would if calcium carbonate were continuously exuded through the cuticle. Furthermore, passage of calcium carbonate in this manner would not be expected on the basis of the structure of the cuticle. There is no indication of any solution of granules on moulting (Müller 1924) or on metamorphosis.

as Keilin (1921) described for the calcospherites in the tubules of *Acidia*, and none are used to impregnate the puparium.

In vertebrates, and in many invertebrates, carbonic anhydrase commonly occurs at sites of calcification and has been found to catalyse the production of carbonate ions, whereby carbon dioxide is made freely available for combination as calcium carbonate. Inhibition of this enzyme by oral administration of a number of materials, including sulphanilamide, has been shown to inhibit egg shell formation in the domestic fowl (Benesch, Barron, and Mawson 1944; Gutawska and Pozzani 1945; Mann and Keilin 1940). Addition of sulphanilamide to the food had no apparent effect on granule formation or on the deposition of carbonates in *L. cuprina* larvae and carbonic anhydrase could not be detected in the tubules or in the larval tissues. This is in agreement with several workers (Florkin 1935; Kreps and Chenykaeva 1942; Sobotka and Kann 1941), who have recorded that carbonic anhydrase is either absent from insects or present in extremely small amounts. If carbonic anhydrase were to occur in insects at all, it might well be expected in the granule-accumulating region of blowfly larvae, but even here it was found to be absent. Its absence favours the elimination of carbon dioxide without hydration and may be responsible for the fact that so little is bound in the granules. Absence of carbonic anhydrase, at least from many insects, renders somewhat less probable the scheme suggested by Wigglesworth (1931) for the formation of uric acid crystals in the malpighian tubules. In this scheme, carbonate is required to displace urate from its cation, resulting in free uric acid.

Alkaline phosphatase is invariably present in quantity at calcification sites in vertebrates (Moog 1946), but does not appear to be present in the cells of the granule region of the tubules, although it occurs abundantly in several other parts of the body (Day 1949).

It might appear then that granule formation occurs in *L. cuprina* as a deposition of salts from solutions concentrated within the lumen of specialized regions of the tubules, without the intervention of at least two of the enzymes which are important in the calcification of the exo- and endoskeletal structures in other animals.

No evidence has been obtained in the present experiments that the granules of *L. cuprina* have any other function than that of storage excretion. While it is true that some respiratory carbon dioxide is bound in the granules, this mode of elimination of carbon dioxide cannot be important to the larva as was suggested by Keilin (1921), since less than 1 per cent. of the amount produced is bound in granule form even under favourable conditions. Also, normal larvae can be obtained which contain few granules, or none, and hence little or no bound carbon dioxide. The fact that normal larvae lacking granules can produce normal adults is good reason to believe that granules are not indispensable to larval physiology. The further fact that any granules which may have accumulated are completely eliminated by the newly emerged adult leads one to the conclusion that granules are merely waste products

stored (by virtue of the absence of peristalsis and of adequate water circulation down the tubules) during larval and pupal life in amount and composition depending upon the nature of the food.

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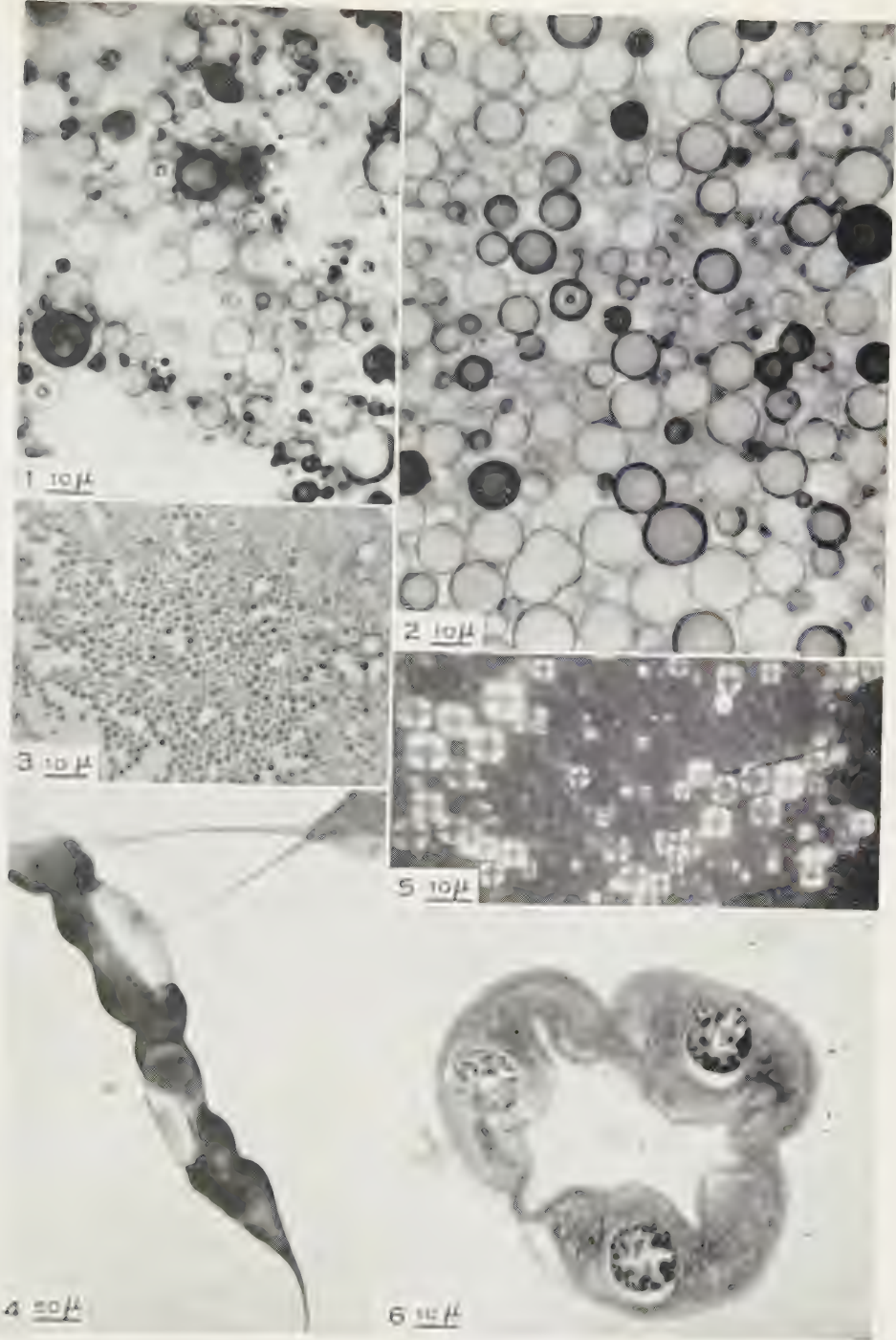
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EXPLANATION OF PLATE 1

- Fig. 1.—Granules, from liver-fed larva, stained for phosphate. Concentric rings within granules in Figures 1 and 2 are mainly due to differences in properties of the various layers and not to purely optical effects.
- Fig. 2.—Granules from larva fed on calcium-enriched artificial medium (24 x Ca as gluconate). Stained with Gallanine blue.
- Fig. 3.—Pupal granules from posterior pair of malpighian tubules.
- Fig. 4.—Posterior blind end of granule-accumulating region showing the terminal filament and two strands of connective tissue attached at one end to tubule and at the other to a branch of an alary muscle.
- Fig. 5.—Granules from larva fed on the artificial medium, photographed under crossed polars.
- Fig. 6.—Transverse section of yellow region of malpighian tubule.





THE REACTIONS OF MOSQUITO LARVAE TO REGULAR REPETITIONS OF SHADOWS AS STIMULI

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Summary

Two factors are involved in the diving reaction of larvae of *Culex fatigans*, Wied., a kinetic stimulus which initiates movement and a directional stimulus. The kinetic stimulus may be a water vibration, a change in light intensity, or a mild electric shock. The directional stimulus is normally a combination of light and gravity.

Regular repetitions in change of light intensity as a stimulus result in a diminution in the degree of response. This rate of diminution is affected by the number of larvae present and their age. The decrease in relation to age is probably to be associated with their increasing oxygen requirements.

I. INTRODUCTION

Mosquito larvae, like many other microphagous feeders, have no discrete organs which could be described as being for either offence or defence. The larval stage in the life cycle is essentially for feeding and growth in size. Food material is stored up in the body during this period for use in the non-feeding pupal stage when extensive organic reconstruction takes place preparatory to the emergence of the imago. The pupal stage is similarly defenceless.

In the absence of weapons for self-preservation in these stages, survival is achieved by the development of defensive behaviour patterns. These consist of vigorous swimming movements which may serve to take them away from the source of danger when danger threatens. Both larvae and pupae, being primarily air breathers, must spend a considerable amount of their time attached to the surface film. In this position, larvae can feed and respire. They do, however, make excursions from the surface to the richer feeding grounds in the detritus at the bottom and they can also, with their feeding brushes, scrape attached diatoms and algae from the surfaces of submerged water plants. Pupae occasionally, too, make rapid and vigorous dives after which they float passively back to the surface film. Whilst at the surface, the animals show considerable sensitivity to changes in light intensity and to water vibrations, reacting to such stimuli by diving energetically with violent lashings of the abdomen. Such "crash dives" of larvae can be readily distinguished from the more leisurely descents to the feeding grounds.

There can be little doubt that this diving reaction is a defensive mechanism. It is, as far as can be seen, the only means of defence possessed by the organisms from predators such as birds, or from predaceous fish or larvae of other

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insects which would create vibrations in the water in their approach. Escape movements are not necessarily away from the source of danger but their direction is determined, as will be shown, by the position of the animal in relation to light and gravity. In the present investigation the nature of the response of larvae has been studied and also their reactions to regular repetitions of changes in light intensity.

The defensive reaction, such as has been described, could then be considered as a response to a biologically relevant stimulus. This work was undertaken to determine how long the response would continue to be evinced. In other words, would they become irresponsive to an often repeated warning stimulus?

II. MATERIALS AND METHODS

Culex fatigans Wied. was used as the experimental animal in all tests described. Wild stocks were used exclusively to ensure that there should be no risk of selection or selective breeding. Egg rafts were collected from an outside aquarium and hatched in the laboratory in filtered water from that aquarium. Larvae used in any one series of tests were hatched from the one egg raft. They were fed on a suspension of "Farex" in water. A few drops of this were added daily to the cultures, the amounts added being determined by the size and rate of growth of the larvae. Sufficient was added to ensure a constant supply of food for the organisms without fouling the water. For newly hatched larvae it was found desirable to make the food very finely particulate by grinding it up with a mortar and pestle but this mode of preparation was unnecessary with larger larvae.

Larvae under test were kept in special vessels made of $\frac{1}{8}$ in. sheet "Perspex" cemented with "Perspex" cement. These vessels have a front face $3\frac{1}{2}$ in. square. The sides slope inwards slightly towards the back so that the rear face is 3 in. wide. The depth from front to back is 2 in. Their capacity is about 200 ml. Containers of this form were adopted in preference to circular or rectangular vessels as they facilitated observation of the movements of the contained animals. These vessels containing animals to be tested were placed each in a black-lined recess, 8 in. wide by 5 in. deep by 22 in. high. A 60-watt pearl lamp enclosed in a metal lamp-house having a circular aperture 2 in. in diameter was suspended in the top part of the recess 12 in. above the water. A white card 3 in. by 3 in. was placed immediately behind the jar so that the larvae would be more readily visible. All tests were carried out in a darkroom. Before any series of tests, the animals were subjected to a steady light for at least half an hour.

In experiments involving electrical stimulation an induction coil with an interrupter in circuit was used. The electrodes were pieces of tinfoil the same size as the sides of the jar and stuck on to these. Large electrodes were used so that whatever the position of the larva in the jar, it could not escape the electric field. No absolute measure was made of the current passing but the peak voltage was recorded on a cathode ray oscillograph so that the same

voltage could be used in successive experiments. It was found that if too powerful a shock were given, the larvae continued to make agitated movements for some seconds after the current had been cut off, while too weak a shock caused them to make a few spasmodic movements without losing contact with the surface film. A strength intermediate between these was used. In all cases described the duration of the stimulus was one second.

The reactions of larvae to regular repetitions of change in light intensity were studied by occulting the light above them for one second in each minute. This was done by wiring the lamp through a cut-out relay which was activated by a 4.5 volt battery in circuit with a contact on the second hand of a large electric clock. The relay was placed on a shelf away from the experimental bench. A few "blank" experiments showed that the larvae were irresponsive to the slight noise of the cut-out and also to a shaded red light which illuminated the dial of the clock. For repetitions of the electrical stimulations, the induction coil and its battery were wired up to the contacts on the clock.

III. NATURE OF THE RESPONSE

Folger (1946) has studied the diving reactions of mosquito larvae and pupae in response to changes in light intensity and mechanical disturbances. He asserts that the downwards movement of the animals as the result of a stimulation involves a negative phototaxis coupled with a positive geotaxis. He postulated "two stimuli which are joined to bring about the descent. One of these is kinetic and induces movement and the other is directive and determines the direction of movement. A kinetic stimulus is associated with time-rate change of the stimulating agent; a directive stimulus with sustained action by it." A change in light intensity or a mechanical disturbance in the water could act as the kinetic stimulus, the direction of movement then being determined by the position of the animal in relation to the light or gravity source. For instance, when the larvae are stimulated by a change in light intensity or a mechanical shock while the light is above them, they go more or less straight down. When the light is from one side, they tend to go down obliquely away from the light, while, when the light is below them their tendency to dive on stimulation is somewhat reduced.

In the present work, an electric shock was used as the kinetic stimulus. Ten third instar larvae were placed in a jar with the light above them. After the usual half hour allowed for acclimatization five shocks, each of one second's duration, were delivered at intervals of one minute. The movements of the larvae are indicated in Table 1.

The experiment was repeated with the same larvae after a lapse of half an hour during which time they were subjected to a steady light but this time with the only illumination below the jar. In this case 12.5 per cent. went to the bottom during the five stimulations. A reversal of the normal position of the light then has an inhibiting effect on the diving reaction. They were then left in darkness for half an hour. At the end of this time a luminescent screen

was placed behind the jar in place of the white card used in the previous experiment. This made the larvae just visible in silhouette. Previous tests had shown that the larvae were unresponsive to light of this low intensity. Five stimulations were again given at minute intervals and 35.7 per cent. of the larvae went to the bottom. In this experiment gravity alone was the directional stimulus. These tests have been repeated several times in various orders, and with different groups of larvae, for example with light below the jar first and above second and so on, but always with substantially the same results, indicating that these differences are real.

TABLE 1
EFFECT OF ELECTRICAL STIMULATION ON LARVAE WITH LIGHT ABOVE

	At Surface Before Shock	Left Surface	Went to Bottom
1	9	9	5
2	9	9	4
3	10	10	6
4	10	10	7
5	10	10	4
Totals	48	48	26
			Average 54.2%

The influence of gravity working alone is also shown by the following experiment. A group of ten third instar larvae were subjected to an occulting light (one second in each minute) until they were completely insensitive (*vide infra*). This took about an hour. Then for five succeeding minutes an electrical stimulus was delivered simultaneously with the occulting of the light. During five stimulations 30.5 per cent. of the larvae went to the bottom. As the larvae had been made insensitive to change in light intensity as a kinetic stimulus the effective one was purely electrical. Further, as the light was occulted during the period of delivery of the shock, the directional stimulus must have been gravity alone. This figure, 30.5 per cent., compares well with 35.7 per cent. obtained with the electrical stimulus in darkness.

IV. EFFECT OF REPEATED STIMULATIONS

When a change in light intensity, i.e. occulting the light, was used as a kinetic stimulus, regular repetition of the stimulus resulted in a fairly rapid diminution in the numbers of larvae which responded to an ultimate condition when all were completely insensitive. The rate of onset of this condition of insensitivity depends on at least two factors, namely the number of larvae present in the group and their age. Many other factors, for example temperature, or amount of food present, will also influence the condition, but these have not as yet been investigated in detail.

The influence of the number of larvae present is illustrated in Figure 1. Here four groups containing respectively 3, 6, 9, and 12 individuals from the

same egg raft and all in their third instar were placed in identical jars. After half an hour of steady illumination, occultation of the light for one second in each minute was started and the movements of the larvae in each jar noted. After ten such occultations, group I (3 larvae) had ceased to respond. Groups II, III, and IV still responded actively but ceased in 34, 50, and 80 minutes

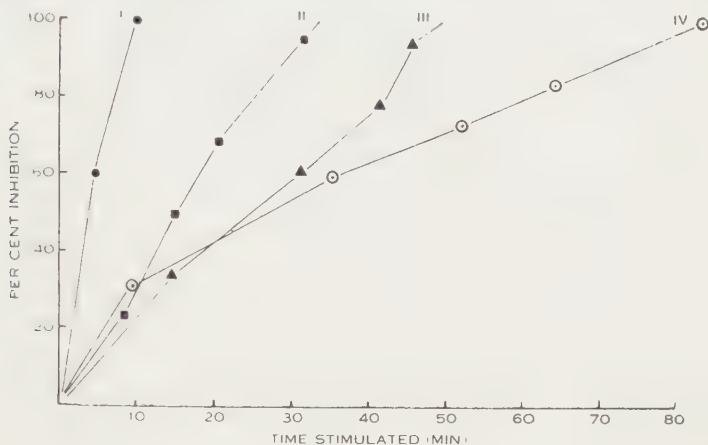


Fig. 1.—Influence of numbers present on the rate of establishment of inhibition to repeated occultations in third instar *Culex fatigans* larvae. Group I, 3 larvae; Group II, 6 larvae; Group III, 9 larvae; Group IV, 12 larvae.

respectively. Ordinates in the graph represent the number of larvae which remained at the surface after stimulation, expressed as a percentage of the number at the surface before stimulation. Each point represents the average results of at least five immediately successive stimulations. The data for repetitions of the experiment with different larvae in groups of three gave an approximate χ^2 of 22.0 on two degrees of freedom for which the probability is less than 0.1 per cent. The figures for replications on both groups of six and nine larvae lead to values of χ^2 significant beyond 0.1 per cent., showing that the increase in inhibition is real for groups of 3, 6, and 9 larvae. A similar degree of probability has been assumed for larvae in groups of 12. Still larger groups, in which the numbers were too large for accurate counting, continued to show some response after several hours of regular occultations.

It would appear then that there is a slower establishment of inhibition in larger groups than there is in smaller ones. This "mass effect" may be caused by a few larvae in the group being more sensitive to stimulation than others. The mechanical disturbance of their movement might act as a kinetic stimulus for others nearby. The larger the group, the greater would be the possibility of there being several of these "sentitives" present, so the longer the reaction of the group as a whole would continue. It was observed that in group IV (12 larvae) and in other cases where a large number of larvae was tested together, when several were gathered in one corner of the jar, some

would respond to the stimulus whereas isolated ones at the other end of the jar remained quiescent. Against this explanation is the also observable fact that when a number of larvae is grouped together, on the surface film, one or two swimming up from below into the middle of the group, even though they nudge several in attaching themselves to the surface film, rarely create a marked disturbance amongst their neighbours. Disturbance alone then is probably not the explanation. It seems then that the additive effect of disturbance and change in light intensity both acting as kinetic stimuli can bring about the continued reaction to stimulation in large numbers.

The effect of age on the reaction is also well marked. In an experiment to test this effect, eighty larvae, newly hatched from a single egg raft, were divided into eight groups of ten. With the exception of the testing to be described, they were kept in as nearly identical position as possible. On the first day group I was subjected to 20 light occultations at intervals of one minute. On the second day, groups I and II were similarly tested, on the third day, groups I, II, and III, and so on. Thus on the eighth day, group I had its eighth trial and group VIII its first. The results are summarized in Figure 2. In this Figure, each heavy vertical line records the number of larvae which remained at the surface on each occultation of the light, expressed as a percentage of those at the surface before the stimulation. A dot



Fig. 2.—Influence of age on the rate of establishment of inhibition to repeated occultations in *Culex fatigans* larvae. Vertical lines represent percentage inhibition. For details of grouping, see text.

on the base line indicates zero inhibition. As the percentage inhibition for the succeeding minute intervals are for the same groups of larvae and are computed from a small number (10 in each group), the results of this experiment are not suitable for a strict statistical analysis. It seems evident from Figure 2, however, that (a) there is little variation between successive groups tested

on the same day and (b) there is an increase in rate and degree to which inhibition is established on successive days.

The latter is attributable to increasing age, the significance of which is demonstrated by an analysis of the results obtained with each group on its first trial. On the basis of the general nature of the experimental results, viz. that data for replications using other larvae are similar, the figures for the minute intervals taken as independent give $\chi^2 = 203.6$ with six degrees of freedom, the probability being less than 0.1 per cent. Six degrees of freedom only are allowed, as the results of the first two days had to be pooled because so few larvae returned to the surface before the succeeding occultations.

These results suggest that no memory factor is involved from day to day. On the eighth day for instance, group VIII receiving its first series of stimulations showed, if anything, a slightly higher rate of establishment of the inhibition than did group I which on that day received its eighth series. The similarity of the graphs on the same day is noteworthy in all cases. The development of the inhibition can then be reasonably associated with the state of morphological and physiological organization of the larvae on successive days, and also with their increasing oxygen requirements with advancing age.

During the experiment all larvae grew to the fourth instar at closely comparable rates. On the first day all were in the first instar, on the second day in the second. On the third and fourth days the third instar had developed, while by the seventh day all but two individuals in group I were in their fourth instar.

For young larvae, the surface-volume ratio is high and most of the oxygen requirements can be met by surface diffusion of dissolved oxygen through the cuticle. As they increase in size, instar by instar, this ratio decreases and more atmospheric oxygen is required to meet the increasing demand. Hence more time must be spent at the surface by older larvae. In Figure 3 are given the percentages of larvae at the surface *before* each stimulation on successive days. Using for each of the eight groups the actual and possible number of larvae at the surface before stimulation, taken over the twenty minutes on the first day of the test in each case and taking the data for the minute intervals as independent, as before, gives a 2×8 contingency table for which $\chi^2 = 807$, $n = 7$ degrees of freedom and $P < 0.0001$ showing the significance of the increase in proportion at the surface with time in days. The figures for the third and fourth days and also those for the fifth to eighth days both lead to values of χ^2 significant beyond $P = 0.001$, showing that the increase in the proportion at the surface during the third and the fourth instars is real.

No figures have been found in the literature so far indicating the oxygen requirements of successive instars, though Fraenkel and Herford (1939) state that *Culex* larvae use twice as much oxygen when they have free access to the water surface as they do when they are kept forcibly submerged in oxygenated water. The authors do not, however, give any indication of the age of the larvae experimented upon. If the results indicated in Figure 3 are in any way significant, there should be a marked increase in oxygen consumption, at

least in the third and fourth instars. Preliminary tests on the oxygen requirements of successive instars indicate that such is the case and further that oxygen consumption increases with age during one instar. Further experiments have shown that whereas first and even second instar larvae can survive when kept submerged by a layer of fine netting, third and fourth instar larvae cannot, unless artificial aeration of the water is resorted to. The increased need for oxygen in older larvae keeps them more at the surface. When stimulated, they are less prone to leave it and if forced down by a strong stimulus, they return more rapidly.

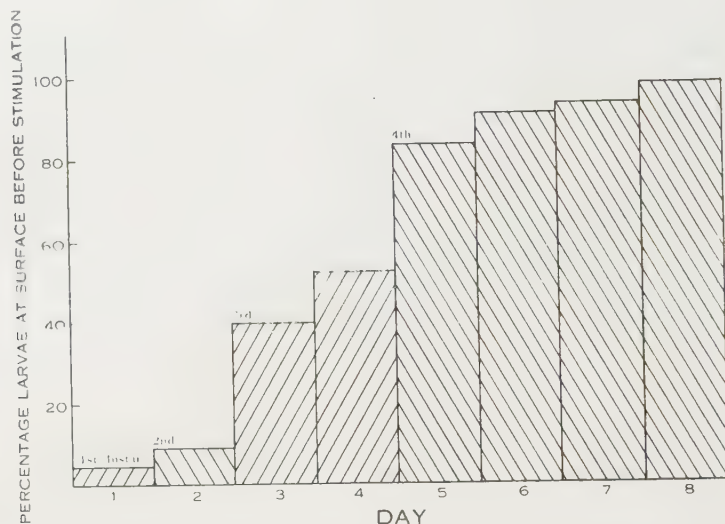


Fig. 3.—Relationship between the age of larvae of *Culex fatigans* and the amount of time spent at the surface.

V. DISCUSSION

Such a cessation in the response to repeated shadows has been noted by several authors working with a variety of animals. Von Uexküll (1897) noted that whereas sea urchins turn their spines towards an appearing shadow they fail to respond after three or four repetitions of the stimulus. Nagel (1896), working with several species of lamellibranchs and gastropods which retract into their shells when a shadow passes over them, states that they are quite unmoved when this has been repeated several times. Yerkes (1906) has recorded conditioning in the annelid worm, *Hydroides dianthus*. These worms retreat rapidly into their tubes when "shadowed" but again, repetition induces inhibition. She found, however, that if a slight tactile stimulus were given immediately after the shadow on several occasions on successive days there was an enhanced response to the shadow alone though after about the eighth day, this response tended to fall off too. Hargitt (1909), also working with the same species, obtained a similar result and showed further that specimens

taken from water of about twenty fathoms or over where shadows were less dense or non-existent, did not respond at all. Mast (1911, p. 249) reports from his own observations that mosquito larvae and tubiculous worms soon fail to respond to ordinary shadows if they are kept in places where shadows frequently occur.

Miller and Mahaffy (1930) have recorded somewhat similar results in the reactions of the holostome cercaria, *C. hamata*. In these larvae a short rapid swim, generally upwards, alternates with a longer period of quiet sinking. If a shadow passes over them or if the light intensity is otherwise reduced, most of them start swimming immediately. These workers found that repetition of "shadowing" at short time intervals (every one or two seconds) resulted in a rapid inhibition. A lapse of longer periods between "shadowings" generally resulted in a maintenance of the response, the longer the time interval between stimuli the greater the probability of there being a response.

Agar (1943) discusses the question of acclimatization to repetitions of a given stimulus under the heading of "Habituation." Quoting from some of his own experimental work on garden snails he shows that the phenomenon depends largely on the strength of the stimulus applied. If this is weak and innocuous, habituation is generally rapid, whereas with strong or painful stimuli, habituation does not occur. He argues then that sensory or motor fatigue could be no more than a small contributory factor as habituation to mild stimuli is more rapid than it is to stronger stimulations. He quotes the experiments of the Peckhams (1887) on spiders whose results parallel to some extent the findings in this paper with mosquito larvae. A spider was stimulated by sounding a tuning fork close to it on its web. It responded by dropping from the web and hanging suspended below it. Shortly, however, it climbed back up its thread on to the web again. Repetitions of this stimulus each time it returned resulted in its dropping shorter and shorter distances each time and returning sooner until after about a dozen trials it ceased to respond at all. Repetitions of this experiment on successive days brought about a further diminution in the response until on the sixteenth day there was no response at all, nor was there, with two minor exceptions, for the succeeding ten days of the experiment.

This habituation over a long period Agar attributes to learning by experience, while the daily habituation might well have a purely physiological basis. For the mosquito larvae the explanation is probably different. The spider, in the course of two or three weeks of the experiment, had not developed appreciably either morphologically or physiologically whereas the larvae during the eight days of the experiments described herein had grown from newly hatched instars to their prepupal condition, involving considerable morphological and metabolic development. The establishment of the inhibition with increasing age might well have had a physiological basis, namely, as has been pointed out above, increased oxygen requirements. Its daily establishment too is more likely to be physiological than psychological. If it is the latter

there is no indication of "memory" from day to day as any group receiving a particular series of stimulations does not show a greater degree of habituation than do those of the same age which have had fewer or no previous experiences of the stimulations. Further, any suggestion that there is "mass psychology" at work in the experiment summarized in Figure 1 presupposes a social unity or bond in the aggregation. Allee (1931) describes such an aggregation as a "synchoropaedium" and Alverdes (1932) as a "conglobation." Neither term connotes anything more than a chance aggregation brought about by purely external influences, so no social unity in the group can be assumed.

If habituation is physiological, sensory fatigue seems to be more probable than motor fatigue. In the discussion on the experiment described in Figure 1, it was pointed out that slower acclimatization of larger groups was attributable to the additive effects of occultation and the presence of "sensitives," giving a more intense total stimulation than occultation alone. Motor fatigue is unlikely as larvae frequently left the surface voluntarily in between and apparently irrespective of the periodic stimulations and moved about in the water quite actively.

A further explanation of the development of the inhibition is possible. If the diving reaction were evinced to every stimulation emanating from a natural environment, feeding, and thus growth rate, would be seriously impaired. Thus its development to a repetition of mild stimuli can be looked on as a behaviour pattern beneficial to survival. This, Agar (loc. cit., p. 115) describes as "negative learning." The same can be said to be the case with the experiments and observations of Nagel on molluscs and Yerkes on *Hydroides*, in which, as with mosquito larvae, feeding is a more or less continuous process and in which frequent interruptions would be detrimental.

A few tests have been carried out on the response of mosquito larvae using slight tactile and vibrational disturbances as kinetic stimulants. They were found to develop an inhibition as they did to shadows though perhaps not as quickly. There is an interesting contrast here with *Cercaria hamata*. Miller and Mahaffy (loc. cit.) found that repetitions of tactile stimuli did not bring about an inhibition, at least not until the larvae were fatigued. The explanation here is that *Cercaria*, once it has come in contact with its host, must continue in its activity to ensure penetration of the tissues and thus survival, but the mosquito larva, as pointed out above, develops the inhibition as a beneficial behaviour pattern.

VI. ACKNOWLEDGMENTS

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OBSERVATIONS ON THE BEHAVIOUR OF PROTEIN IN PAPER PARTITION CHROMATOGRAPHY

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Summary

A study has been made of the behaviour in partition chromatography on filter paper, by the method of Consden, Gordon, and Martin (1944), of several proteins commonly occurring in biological fluids. It is shown that the protein in such fluids may appear as a ninhydrin-reacting spot in a characteristic position on the chromatogram, and present a problem in identification.

The proteins tested have an R_F value in phenol of 1, and in collidine of 0. After their movement in phenol their subsequent elutability in distilled water and in phenol is affected by such factors as the method used for removing the phenol, and whether the filter paper is used as supplied, or whether contaminating substances present in the filter paper have been removed by prior washing with water.

It has been found that amino acids and peptides are not split off proteins during their movement in phenol and that the area of the ninhydrin-reacting spot given by the protein after a phenol chromatogram is approximately proportional to the concentration of the protein in the test sample.

I. INTRODUCTION

In applying the method of partition chromatography on filter paper first described by Consden, Gordon, and Martin (1944) to a study of the ninhydrin-reacting substances in biological fluids, the authors have found that chromatograms of such material frequently show a ninhydrin-reacting spot in a characteristic but unusual position. This has been shown to be due to protein in the test sample. The protein moves as rapidly as the front in phenol, and subsequently not in collidine. The movement of protein has not so far been referred to in the published work dealing with paper partition chromatography. For example, no position is assigned to protein in the "map of the spots" published by Dent (1948) although 60 ninhydrin-reacting substances are listed.

The results are reported in three sections. The position of a ninhydrin-reacting substance, found in chromatograms of plasma and serum "deproteinized" by alcohol, and the tests which identify it as protein, are described in the first section. In the second section two practical problems are considered, namely whether the proteins carried down to the furthest part of the chromatogram in phenol can then be eluted from the filter paper strip with distilled water, or phenol, and secondly, whether during its downward movement in phenol, amino

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acid or peptide fractions are split off the protein and distributed over the length of the chromatogram. In the third section some further observations are reported on protein behaviour which are relevant to the technique of paper partition chromatography and its possible uses.

II. EXPERIMENTAL

(a) *Materials and Methods*

The chromatographic method of Consden, Gordon, and Martin (1944) was used, the solvents being phenol saturated with water (the atmosphere containing NH_3 and HCN), and a mixture of equal parts of *s*-collidine and 2,4-lutidine saturated with water.

Preparation of blood plasma and serum.—Blood plasma and serum of normal fasting subjects were treated with absolute alcohol at room temperature (final concentration 70 per cent. v/v) to precipitate the protein, centrifuged, and the supernatant concentrated *in vacuo*, to remove the alcohol. The supernatant was then “desalted” (Consden, Gordon, and Martin 1947) and concentrated *in vacuo* to 1/15 the original plasma volume. A 20 μl . aliquot of this prepared plasma was submitted to chromatography. In the subsequent text the ninhydrin-reacting substance found in chromatograms of the prepared plasma and which is shown to consist of protein is referred to as Fraction P.

Protein solutions.*—Solutions of the following purified proteins were subjected to chromatography:

- (1) A 2 per cent. solution in distilled water of human serum albumin (Fraction V Cohn's method).
- (2) A 2 per cent. solution of human serum globulin (Fractions II and III Cohn's method) in dilute sodium chloride solution.
- (3) A 3 per cent. solution of egg albumin in distilled water.
- (4) A 2 per cent. solution of human sero-mucin in N/100 NaOH.

(b) *Elution Experiments with Fraction P and with Known Proteins after Chromatography with Phenol*

Unidimensional chromatograms of “prepared plasma” and the protein solutions were run a distance of approximately 40 cm. in phenol. The phenol was removed in one of three ways—heating to 100°C. for 1 hour, air-drying at room temperature with an electric fan for 24 hours, or rapid removal of the phenol with acetone. Washed and unwashed filter paper was also used since in a preliminary communication (Wynn 1949) it was demonstrated that filter paper may contain a peptide-like contaminant, and also other substances, mainly inorganic salts and carbohydrates. These latter substances, carried

* The sero-mucin was prepared by Dr. J. F. McCrea by a modification of Rimington's method (McCrea 1948). We are indebted to Prof. F. M. Burnet, Walter and Eliza Hall Institute, for this specimen. The human albumin and globulin were kindly supplied by Mr. F. J. Dempster, Commonwealth Serum Laboratories, and the egg albumin by Dr. A. Pound, Department of Pathology, University of Melbourne.

down in the phenol with the protein being tested, could possibly affect its elutability. After removal of the chromatographic solvent the paper was reversed and the eluting solvent—either distilled water or phenol saturated with water—run in the reverse direction to practically the full length of the paper. The paper was then dried, developed with ninhydrin, and the size and intensity of colour of the ninhydrin-reacting material compared with a control, which was the corresponding unidimensional chromatogram which had not been subjected to the elution procedure.

(c) *The "Cutting-out" Experiments with Known Proteins*

The unidimensional method of Consden, Gordon, and Martin (1947) and Dent (1947) was used. Fourteen 20- μ l. drops of the protein solution being tested were placed side by side on a sheet of *washed* Whatman No. 4 filter paper and run 40 cm. in phenol. The phenol was removed by heating to 100°C. for 1 hour. Four strips, each approximately 10 cm. wide, were cut transversely across the full width of the paper from the origin to the phenol front. The ninhydrin-reacting material was located in each case on the strip adjacent to the solvent front. The strips were then eluted with distilled water. The eluates were dried *in vacuo*. The residues were taken up in 0.1 ml. of distilled water and 20- μ l. aliquots run as chromatograms in phenol. The residual eluates were hydrolysed (6N HCl 105°C. for 24 hours) and unidimensional chromatograms of the whole of the hydrolysed material were run.

III. RESULTS

(a) *A Complex Ninhydrin-Reacting Substance (Fraction P) in Alcohol-Prepared Blood Plasma*

Two-dimensional chromatograms of the blood plasma or serum prepared as described above consistently revealed, apart from a number of amino acids, a ninhydrin-reacting substance (Fraction P) which moved to the edge of the phenol front but did not move in collidine (Plate 1, No. 11). It corresponded in position to Dent's (1948) "nephrosis peptide," a substance found by him in the "blood and deproteinized urine" of a nephrotic patient and occasionally found in traces in other urines. Dent does not give details of the method he used to deproteinize the blood or urine so that the substance found in all the plasmas and sera examined by the authors cannot further be compared with his.

The Identification of Fraction P.—Fraction P was isolated on the paper by running unidimensional chromatograms in phenol, removing the phenol by heating to 100°C. for 1 hour, and then running collidine in the reverse direction to carry back any amino acids or simple peptides. Fraction P could now be shown to be isolated as a single ninhydrin-reacting spot extending about 1½ in. from the edge of the phenol front towards the origin. It could not now, however, be eluted from the paper with distilled water, phenol saturated with water, or glacial acetic acid. Its insolubility suggested denaturation, and that

Fraction P was not a polypeptide of small molecular weight but most likely a protein. The following evidence supported this assumption:

- (1) Fraction P was not removed from the prepared plasma by 24 hours dialysis against distilled water, indicating that it was a large molecule.
- (2) Fraction P disappeared on acid hydrolysis of the prepared plasma (6N HCl at 105°C. for 24 hours) and chromatography then showed a large increase in the number and amounts of amino acids. Since deproteinized plasma contains very little of the conjugated forms of amino acids (Christensen and Lynch 1946), the large increase in free amino acids on hydrolysis of the prepared plasma strongly suggested that some protein had not been precipitated by the alcohol treatment. The disappearance of Fraction P on hydrolysis is consistent with the belief that it was this protein.
- (3) Concentrations of alcohol from 60 to 85 per cent. v/v were used to precipitate the plasma proteins. The amount of material in each specimen of plasma behaving as Fraction P varied inversely as the alcohol concentration, the greatest amount being found with 60 per cent. alcohol, and none being detectable with 85 per cent. alcohol.
- (4) Chromatograms of the known protein solutions were run. All the proteins tested moved rapidly in phenol to a position similar to that noted for Fraction P. Subsequently they did not move in collidine. When collidine was used as the first solvent, albumin and globulin showed no movement but with sero-mucin and Fraction P, on spraying with ninhydrin, a short comet effect was obtained due to movement of portion of the protein, the bulk remaining at the origin. After the collidine run none of the proteins moved subsequently in phenol.
- (5) The effect of prior heat denaturation upon the movement in phenol of Fraction P and of the known proteins was studied. The test solution was dried on the paper by heating to 100°C. for 1 hour in order to denature the protein. The subsequent chromatograms showed that the bulk of the protein was now retained at the origin where it gave a strong ninhydrin reaction, but a variable portion moved with the phenol front. Heat denaturation produced less retention of material at the origin with sero-mucin and Fraction P.

These results supported the conclusion that Fraction P, found in the filtrate of blood plasma and serum, was portion of the plasma protein which had not been precipitated by treatment with 70 per cent. alcohol.

(b) The "Elutability" of Proteins after Chromatography with Phenol

The results of the experiments are presented in Tables 1 and 2. Various degrees of elutability are described depending upon the movement of the protein in the second solvent:

- + + + + + The protein moves *en bloc* in the second solvent and as rapidly as the solvent front.
- + + + + + The protein moves *en bloc* but more slowly than the second solvent.
- + + + + The bulk of the protein moves as rapidly as the second solvent but leaves a detectable trace behind in the original position.
- + + + Approximately half the material moves with the second solvent front, and half remains in the original position.
- + + Some material moves rapidly but the bulk is immobile and remains in the original position.
- No visible movement or alteration in the size and colour of the ninhydrin-reacting spot.

TABLE 1
CHROMATOGRAPHY WITH PHENOL-NH₃-HCN AND ELUTION WITH DISTILLED WATER

Filter Paper	Method of Removal of First Solvent	Protein "Elutability"			
		Fraction P	Sero-mucin	Albumin	Globulin
A	Heat	—	—	—	—
B	Heat	—	—	—	—
A	Air dried	—	—	—	—
B	Air dried	+ +	—	—	—
A	Acetone	+ + + +	+ + + + + +	—	—
B	Acetone	+ + + + + +	+ + + + + +	+ +	—

* Whatman No. 4. A—used as supplied; B—used after washing with water.

From Table 1 it can be seen that the four proteins tested could not be eluted with distilled water when the phenol chromatographic solvent was removed by heating the paper to 100°C. for 1 hour, or by drying in air, but removing the phenol with acetone favoured the elution of Fraction P and sero-mucin. Using washed filter paper also had a demonstrable effect in increasing the elutability of some of the proteins.

Table 2 demonstrates similarly that the elutability of the proteins, when phenol is used both as the chromatographic and eluting solvent, may be affected by the method of removing the chromatographic solvent, and by using washed or unwashed filter paper.

"Cutting-out" experiments.—With all the proteins tested the same result was obtained. No ninhydrin-reacting material was shown in either the unhydrolysed or hydrolysed eluates. Therefore, there was no evidence that amino acids or peptides could be split off the four proteins tested during their movement downward in phenol.

(c) Further Observations upon the Movement of Protein

The following procedure sufficed to immobilize the protein at the origin, enabling a normal amino acid chromatogram to be obtained from a mixture of protein and amino acids.

- (1) "Collidine" was used as the first solvent (Plate 2, Fig. 1).
- (2) When a unidimensional phenol chromatogram was required the chromatogram was developed first with collidine until the front had passed over the test material at the origin. The collidine was then removed by thorough heating—for example, 3 hours at 80°C. A subsequent phenol chromatogram showed the protein immobilized at the origin and normal separation of the amino acids.
- (3) The test material was heat-denatured after being applied to the filter paper. This treatment rendered the bulk of the protein immobile, the material which still showed rapid movement in phenol being insufficient to cause noticeable interference with the R_F values of the amino acids, provided the original protein concentration of the test material was not much in excess of 2 per cent.

TABLE 2
CHROMATOGRAPHIC SOLVENT AND ELUTING SOLVENT: PHENOL-NH₃-HCN

Filter Paper ^o	Method of Removal of First Solvent	Protein "Elutability"			
		Fraction P	Sero-mucin	Albumin	Globulin
A	Heat	++	—	—	—
B	Heat	+++++	—	—	—
A	Air dried	+++++	+++	—	+++++
B	Air dried	+++++	+++	+++	+++++
A	Acetone	+++++	+++++	+++++	+++++
B	Acetone	+++++	+++++	+++++	+++++

^o Whatman No. 4. A—used as supplied; B—used after washing with water.

When solutions of albumin, globulin, and sero-mucin (the only proteins tested) were run in phenol the area of the ninhydrin-reacting spot given by the protein was roughly proportional to the concentration of the protein in the test sample (Plate 2, Fig. 2). This was true provided the concentration was not too great, since with concentrations of the order of 10 per cent. the ninhydrin-reacting material extended from the origin to the edge of the phenol front. A small ninhydrin-reacting spot a few millimetres in length at the edge of the phenol front represented, with the proteins tested, a concentration of protein less than 0.5 per cent. Protein concentrations smaller than this may be undetectable chromatographically. These observations suggest a possible usefulness of the chromatographic method for giving a reasonable approximation to the concentration of the protein in the test sample, only 20 μ l. of the solution being required.

It is worth noting that a small ninhydrin-reacting spot at the phenol front is likely to be obscured by the reddish, discoloured areas which normally occur at the front but which can be considerably reduced by attention to a number of details (Draper and Pollard 1949). When such care is taken, close examination of the unidimensional chromatogram of normal urine frequently shows the presence of a small ninhydrin-reacting spot at the edge of the phenol front. It is due possibly to the trace of protein which may occur in normal urine and which is not precipitated by trichloroacetic acid (Beckman *et al.* 1943). This spot disappears on hydrolysis of the urine.

IV. DISCUSSION

Partition chromatography on filter paper has become a widely used technique for the identification of ninhydrin-reacting substances. In applying this method to protein-containing solutions it has been generally accepted (e.g. Dent and Schilling 1949) that since proteins present in concentrations greater than about 0.5 per cent. cause distortion of the chromatogram, it is necessary to remove the protein before chromatography. However, it is clear from the results reported above that at least one method of protein precipitation employed in chromatography—namely, 70 per cent. alcohol—may not completely remove the protein. Moreover, solutions such as urine and cerebrospinal fluid normally contain such a small amount of protein that its prior removal is not usually undertaken. However, it is often necessary to concentrate these “protein-free” solutions ten or more times prior to chromatography so that the protein concentrations of the test material applied to the paper may then not be negligible. It is therefore of importance to know where small amounts of protein will appear on a chromatogram.

The authors’ observations indicate that with the types of protein tested a position on the chromatogram can be predicted for the protein. It moves as rapidly as the phenol front and then does not move in collidine. With collidine as the first solvent it does not move, or moves very slightly, and then does not move in phenol. It is unlikely that the movement observed represents true partition chromatography owing to the macromolecular size of the solid components, adsorption effects probably being of major consequence (Martin and Synge 1941; Synge 1946). Protein, however, is not the only ninhydrin-reacting substance which moves as rapidly as the phenol front. The authors have observed the same type of movement with peptone, polypeptides such as thyrotrophic hormone, pancreozymin, and ninhydrin-reacting polypeptides obtained during the preparation of secretin (unpublished observations). Reference to a substance “nephrosis peptide,” described by Dent (1948), occurring in a similar position to that noted by us for protein, has already been made. Dent also noted a substance in “other urines” which “moves to the same place as nephrosis peptide.” The possibility that this substance and “nephrosis peptide” are protein that has not been removed by the deproteinizing procedure has to be considered.

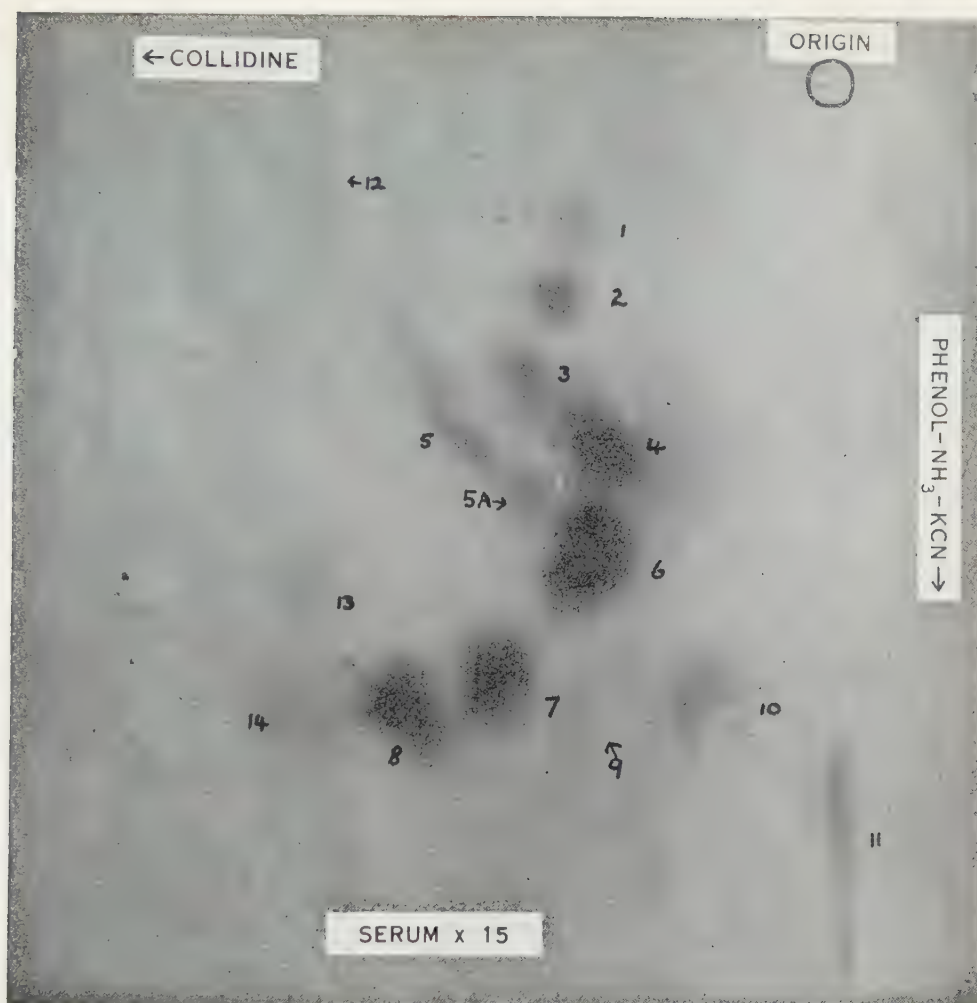
It was shown in Section III(c) that with the proteins tested, concentrations of less than 0.5 per cent. may not give a visible ninhydrin-reacting spot. Consequently the presence of protein of this order of concentration in the test sample may not be suspected, but hydrolysis of such amounts of protein would certainly reveal amino acids readily detectable on chromatography. Since the "cutting out" technique for isolating ninhydrin-reacting substances from a mixture (Consden, Gordon, and Martin 1947; Dent 1947) involves running several drops of the solution side by side on one sheet paper, if the test material contains even small amounts of protein, the total amount of protein on the paper may not be inconsiderable. Therefore it was necessary to examine the behaviour of protein further, to determine whether the protein itself, or amino acid or peptide fractions split off the protein, could be eluted from the filter paper by distilled water, after the protein solutions had been run in phenol, and the phenol removed by heating to 100°C. for 1 hour. The experimental results described in Section II demonstrated that protein does not contribute to the elutable material under the experimental conditions outlined above. But when the phenol is removed by air-drying, or with acetone, some of the protein may be eluted with water and hence contribute to the amount of material recovered from the region of the phenol front in "cutting-out" experiments such as those described by Wynn (1949).

V. ACKNOWLEDGMENTS

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Note: The origin is outlined in ink.

Chromatogram of blood serum.

1. Aspartic acid. 2. Glutamic acid. 3. Serine. 4. Glycine. 5. Taurine. 5A. Threonine.
 6. Alanine. 7. Valine. 8. Leucines. 9. Proline. 10. Lysine-arginine. 11. Protein. 12.
 Cysteic acid (trace). 13. Tyrosine. 14. Phenyl-alanine.

FOSSIL BANKSIEAE FROM YALLOURN, VICTORIA, WITH NOTES ON THE MORPHOLOGY AND ANATOMY OF LIVING SPECIES

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Summary

The morphological and anatomical features of six types of fossil leaves from brown coal at Yallourn, Victoria, have been described. Comparison with similar features of living species has shown that all are extinct members of the tribe Banksieae of the family Proteaceae. A new genus *Banksieaephyllum* has been instituted to include these fossil leaves. The suggestion has been made that the identification of fossil leaves with either *Banksia* or *Dryandra* is impracticable.

Fruiting cones of *Banksia* from Tertiary deposits are figured and described. The morphology and anatomy of a leaf, *Phyllites yallournensis*, have been investigated.

The chief anatomical features of the leaves of 53 species of *Banksia* and 51 species of *Dryandra* are considered.

I. INTRODUCTION

This paper is mainly concerned with the description of some Victorian Tertiary leaves, the morphological and anatomical features of which suggest affinities with the tribe Banksieae of the family Proteaceae. Previous identifications of fossil leaves with either *Banksia* or *Dryandra* (see Kausik 1943), the two closely allied genera that comprise this tribe, have not, as far as the authors are aware, been supported by anatomical investigations. The advantages of information from such a source, as well as the question of the reliability of morphological characters as guides to the identity of fossil Banksieae, will be discussed. The results of a detailed study of the cuticles of living Banksieae, made in an attempt to determine the affinities of the fossil types, have been presented in condensed form in Tables 1 and 2. The principal characters will be individually considered in the text.

II. MATERIAL AND METHODS

The leaves were collected from the brown coal deposits exposed in the Yallourn North and Yallourn open cuts in south-eastern Victoria. These deposits are being worked by the State Electricity Commission and one of the types was collected by Mr. A. J. H. Adams of that organization. Agreement as to the precise age of these beds has not been reached but it is generally believed to be Oligocene.

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The fossils are preserved as "mummies" and retain much of their original structure. Treatment with a 12 per cent. solution of sodium hypochlorite was found to be the most satisfactory method of maceration but the use of Schultze's solution was sometimes found advantageous. The cuticles thus freed and cleared were washed in water, stained with safranin, and mounted in glycerine jelly. Cuticular preparations of living species were obtained in the same way, but, when herbarium material was used, the leaves were boiled in water prior to immersion in the macerating solution.

Transverse sections, which provide instructive details regarding the internal anatomy of the leaves, were prepared by the use of the paraffin method.

III. GENERAL MORPHOLOGY OF LEAVES OF BANKSIEAE

In 1925 Deane drew attention to the close similarity in external form that exists between leaves of *Banksia* and *Dryandra*. He pointed out that "the so-called *Dryandra* form of leaf, as usually understood, is not peculiar to that genus but occurs also in many species of *Banksia*." His illustrations give clear support to this statement. Referring to the identification of isolated fossil leaves from brown coal deposits at Morwell (the locality now known as Yallourn North) he remarked that "It may be that the fossil leaves with this character should be placed under *Banksia* and not under *Dryandra* at all . . . Following Professor Ettingshausen's lead, however, I have called the serrated leaves *Banksia* and those possessing more or less triangular pinnae *Dryandra*."

Since the results of the present investigation suggest that a revision of Deane's policy concerning the determination of fossil leaves of Banksioid form is desirable, it may not be out of place to elaborate further the difficulties of separating, on external form alone, the leaves of these two genera.

Species having simple leaves with entire margins are rare in *Dryandra* and more numerous in *Banksia*, but the long, narrow, linear leaves with entire margins of *B. tricuspis* have their counterpart in those of *D. speciosa*. As examples of broadly obovate, prickly-toothed leaves, those of *B. coccinea* and *D. cuneata*, or *B. goodii* and *D. praemorsa* may be cited. More deeply lobed leaves of essentially similar form, although of somewhat different size, are found in *B. victoricae* and *D. kippistiana*. Of the pinnate types, *B. speciosa* and *D. formosa*, also *B. dryandroides* and *D. baxteri*, have the same general form, again with the *Banksia* species as the larger. Furthermore, whilst the bipinnatifid character of the leaves of *D. bipinnatifida* cannot be exactly matched in any species of *Banksia*, it is approached to some extent by *B. repens*.

The venation is similar in both genera. The midrib and veins are usually conspicuous and always more so on the lower surface, but they may be obscured to some extent by the hairy covering. The secondary veins arise at a wide angle and run directly to the margin of the leaf, the larger ones passing to the tips of the serrations or pinnae when these are present.

IV. CUTICULAR STRUCTURE OF LIVING SPECIES OF BANKSIEAE

The close similarity between *Banksia* and *Dryandra*^{*} expressed in the external morphology of their leaves is further emphasized when their cuticles are compared. All the main characters and their variants occur in both genera, and a constant distinguishing feature has not been discovered.

(a) *Position of the Stomata*

The leaves of all members of the Banksieae are bifacial, the stomata being confined to the lower surface. In most species the stomata are situated on the sides and bases of small pits or depressions and are accompanied by numerous hairs which develop from the interstomatal epidermal cells. These stomatal pits early attracted the attention of botanists of whom, according to Jönsson (1878), Schleiden was the first.

The breadth and depth of the stomatal pits vary considerably from species to species, and have no constancy in the individual sections of either genus. In five of the species of *Banksia* (Table 1) belonging to the section *Oncostylis* (Bentham 1870) the stomata are diffusely arranged in longitudinal grooves one on either side of the midrib. In the remaining members of the same section the lamina between the margin and the midrib is wider and flatter, so that the stomata, as well as being diffuse, are superficially placed.

Three species of *Dryandra*, namely *D. kippistiana* of the series *Floribundae* and *D. serra* and *D. foliolata* of the series *Concinnae* (Bentham loc. cit., p. 564), also have their stomata superficially placed, but in none are stomatal grooves developed.

(b) *The Stomata*

The stomatal characters of *Banksia* and *Dryandra* were first investigated by von Mohl (1833) who found them to be in agreement with those of several other genera of the Proteaceae (Solender 1908, p. 712).

In this basic type of stoma, from which the authors have found no deviation in the Banksieae, the two guard cells are accompanied by a pair of subsidiary cells arranged parallel to the pore. The guard cells are usually slightly raised above the epidermal cells and their cuticular ridges are prominent.

(c) *Hairy Covering*

The leaves are never completely devoid of hairs in any species of *Banksia* or *Dryandra*. Entire hairs may be either sparsely developed or completely absent from the upper surface of mature leaves. When the latter condition prevails, hair-bases, from which the hair proper has been shed during the development of the leaf, can be observed on detached cuticles of most species (Tables 1 and 2). On the lower surface, on the other hand, hairs, apart from those in the stomatal pits, are sometimes so plentiful that they form a thick woolly felt, as, for example, in *B. robur* and *D. concinna*.

The hairs of the Banksieae have been described and illustrated by several earlier writers (Engler 1889; Hamilton 1907; Haberlandt 1909). They are simple and bicellular. Typically each consists of a short, thick-walled basal cell

* Chatterway (1948a, 1948b) has shown that the woods of these genera are indistinguishable; the same also applies to the pollen grains.

connected with one or more epidermal cells and a terminal, thick-walled, pointed hair-cell. Variations in the shape and proportions of these two components have enabled the following types, illustrated in Figure 1, to be distinguished:

Type (a).—Consists of a cylindrical basal cell of variable length (up to $45\ \mu$ in *B. sceptrum* and *B. hookeriana*) with a diameter of $8\text{--}12\ \mu$ throughout, and a long, thick-walled, often spirally coiled terminal cell the diameter of which ($5\text{--}6\ \mu$) is generally considerably less than that of the basal cell.

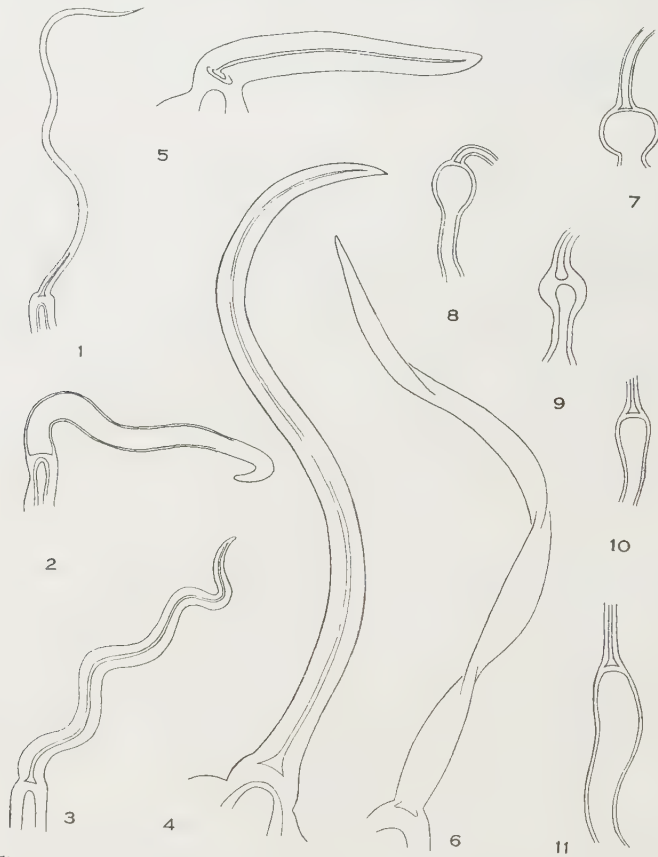


Fig. 1.—Types of hairs found in the tribe Banksieae. $\times 270$.
 1. Type a. *Banksia marginata*. 2. Type b. *Banksia goodii*.
 3. Type b. *Dryandra quercifolia*. 4. Type b. *Banksia robur*.
 5. Type c. *Dryandra praemorsa*. 6. Type d. *Banksia robur*. 7. Type
 e. *Banksia verticillata*. 8. Type e. *Banksia sphaerocarpa*. 9. Type
 e. *Banksia tricuspis*. 10. Type f. *Dryandra stuppea*. 11. Type f.
Banksia hookeriana.

Type (b).—The basal cell is thick-walled, $10\text{--}20\ \mu$ wide throughout; occasionally, as in *B. robur*, measuring as much as $28\ \mu$. The terminal cell is usually thick-walled, about $8\text{--}22\ \mu$ wide, of variable length, angularly bent, loosely coiled, or almost straight, the surface sometimes striated.

Type (c).—The basal cell, 19-60 μ wide, has a strongly thickened wall and a correspondingly reduced cavity; the terminal cell is short, straight or nearly so, and very thick-walled. This type is associated with the upper epidermis or midrib and is well seen in such species as *B. prionotes* and *D. bipinnatifida*.

Type (d).—The basal cell is short, 14-25 μ wide and thick-walled; the long terminal cell, about 12-15 μ wide, has a rather thin wall which is twisted in a loose spiral as in the hairs of *Gossypium*. Such hairs occur in *B. robur*, *D. ashbyi* etc.*

Type (e).—The basal cell of this type is differentiated into a globular "head," 10-36 μ in diameter, to the centre of which the terminal hair-cell is attached, and a more or less extended "stalk" approximately 8.5 μ wide. The total length of the basal cell varies from 25-56 μ and its wall is either thin or thickened to varying degrees. The terminal cell is narrow, usually thick-walled, and spirally coiled. Examples of this type are found in *B. verticillata* and *D. praemorsa*.

Type (f).—The basal cell is club-shaped, frequently thin-walled, and of variable length, ranging from 28 μ in *D. nobilis* to 90 μ in *B. hookeriana*; the terminal hair-cell, which is broadly attached, is thick-walled and spirally coiled.

(d) Epidermal Papillae

This term refers to conical extensions of the outer walls of epidermal cells into which the cell-lumen passes (Fig. 2; Plate 8, Fig. 58). Such papillae are developed in only a few species of *Banksia* and then are restricted to those



Fig. 2.—Epidermal papillae of *Banksia quercifolia*. x 400.

cells adjacent to or abutting on the stomatal pits (*B. quercifolia*) or grooves (*B. pulchella*, *B. meisneri*). Each cell has one centrally placed papilla. Epidermal papillae have not been observed in any species of *Dryandra*, but their infrequent occurrence in *Banksia* makes them of little systematic value. Epidermal papillae have been recorded by Jönsson (loc. cit., Table 3, Fig. 36) as present in the lower epidermis of *Embothrium coccineum* Forst., but in this species the whole outer wall is involved and not only the central portion of it, as in *Banksia* spp.

* Hairs of this type occur on the bracts of cones of *Banksia integrifolia* and *B. marginata*.

(e) Cuticular Papillae

These are verrucose thickenings of the outer cutinized layers of the epidermal cell walls (Fig. 3). They have been observed in 96 per cent. of the species of *Dryandra*, the two species from which they appear to be absent being *D. speciosa* and *D. conferta*, and in 38 per cent. of the species of *Banksia*. They may be distributed on both surfaces of the leaf, as in *B. baxteri* and *D. praemorsa*, but are usually only on one. If the number of papillate cells in the lower epidermis is small or the papillae are not clearly defined, their presence can usually be detected in the vicinity of the stomatal pits.

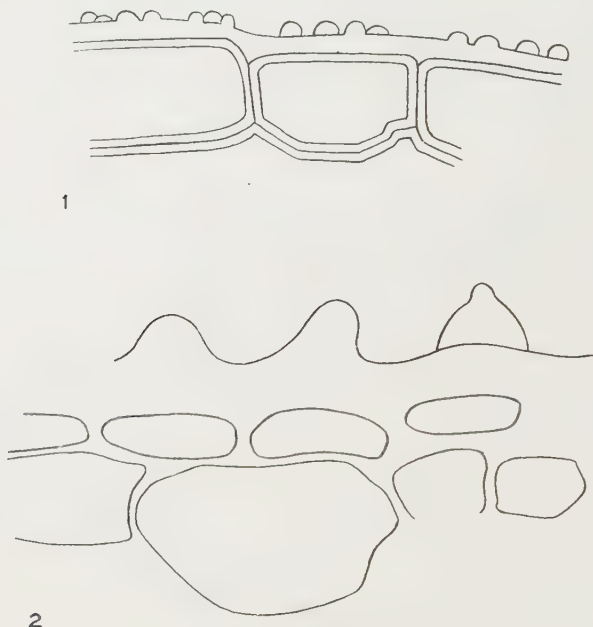


Fig. 3.—Cuticular papillae.

1. T.S. Upper epidermis of *Dryandra foliolata*. x 320.
2. T.S. Upper epidermis of *Banksia verticillata*. x 320.

The number of papillae per epidermal cell is generally proportional to their size and may vary from one (*B. hookeriana*) to thirty or even more (*D. squarrosa*). Their outline in surface view is also variable; they may be circular, oval, or in elongated cells, such as those covering the midrib and secondary veins, ridge- or streak-like. Usually cuticular papillae are restricted, as in *D. praemorsa* (Plate 8, Fig. 59), to those epidermal cells from which hair-bases are absent, but in heavily papillate forms such as *D. kippistiana* and *D. foliolata* (Plate 8, Fig. 60) they are also present on the cells supporting the hair-bases.

(f) Epidermal Parenchyma

(i) *Lower Epidermis*.—The parenchyma which lines the stomatal pits is usually different from that between the pits and covering the veins. The cells

associated with the stomata have thin, slightly curved lateral walls with a large number of pits. The cells of the non-stomatiferous parenchyma, on the other hand, are less uniform, have thicker walls and in some forms, such as *B. attenuata* and *B. baueri*, are of two sizes, small cells which support hair bases and larger ones without hairs (Plate 8, Figs. 61, 62). Their lateral walls are generally straight or slightly curved, thin or as much as 8 μ thick, and pitted or unpitted according to the species.

(ii) *Midrib*.—The cells covering the midrib are more or less elongated and have straight, moderately thickened lateral walls which may or may not be pitted.

(iii) *Upper Epidermis*.—In some species (*B. elderiana*) the cells of the upper epidermis are identical with those of the lower surface. More often there are small differences such as a greater uniformity, a somewhat larger size, more regular form, and, on the whole, thicker lateral walls which may be pitted or unpitted.

V. INTERNAL ANATOMY OF THE LEAVES OF BANKSIEAE

In addition to symmetry, two structural features have been used by Jönsson (loc. cit., p. 49) to distinguish leaves of *Banksia* and *Dryandra* from those of other proteaceous genera. They are the frequent presence of a hypodermis and, in most species, the association of anastomosing plates of sclerenchyma with the vascular bundles of the leaf.

Hypodermis.—In the leaves of most species of *Banksia* and *Dryandra* there are one or two layers of cells between the epidermis and the mesophyll tissue. These may constitute a true hypodermis and the cells be either larger (*D. squarrosa*) or smaller (*D. ashbyi*) than those of the epidermis, or they may be fibrous in character and belong, as asserted by Jönsson, to the bast proper.

In three species of *Dryandra*, namely *D. kippistiana*, *D. concinna*, and *D. foliolata*, neither hypodermal nor fibrous layers are developed.

Sclerenchyma.—The leaves of most Banksieae are highly sclerenchymatous. This is partly the result of the rich development of fibrous cells around the vascular bundles of the midrib and lateral veins and partly the presence of characteristic "vertically transcurrent plates" of sclerenchyma which reach the hypodermis when one is present (Solleder 1908, p. 770). This sclerenchymatous network has been observed during the present investigation, in most species of *Banksia* and *Dryandra*, the only exceptions being those species of *Banksia* which comprise the section *Oncostylis*. Its absence from *B. pulchella* and *B. littoralis*, both members of this section, was recorded earlier by Jönsson (1878) who also showed that, with the exception of a few species of *Hakea* and *Synaphea*, it does not occur in other members of the Proteaceae. Taken in conjunction with other characteristics, therefore, this feature seems to be of value in the separation of the Banksieae from other tribes of this family, and has been used for this purpose in the section dealing with fossil species.

Outer walls of epidermal cells.—These are invariably thick and highly cutinized on both surfaces. This is particularly noticeable on the upper surface where the thickness is frequently considerable. Amongst species of *Banksia* a maximum thickness of $28\ \mu$ is seen in *B. media*, a minimum of $5.5\ \mu$ in *B. marginata*, *B. serratifolia*, *B. ornata*, *B. sphaerocarpa*, *B. spinulosa*, *B. meisneri*, and *B. pulchella*. In *Dryandra* a maximum thickness of $25\ \mu$ occurs in *D. preissii*, *D. pteridifolia*, and *D. plumosa*, while the minimum, $5.5\ \mu$, occurs in *D. floribunda*. The material from which these figures have been derived came from natural habitats, so that there is no reason to think that the approximate measurements given are in any way abnormal.

VI. SALIENT CHARACTERS OF LEAVES OF THE BANKSIEAE

The preceding survey of morphological and anatomical features of the leaves of living members of the tribe Banksieae has shown:

- (1) That there is no feature by which leaves of *Banksia* can be safely distinguished from those of *Dryandra*.
- (2) That some features are constant in the tribe:
 - (a) A pair of subsidiary cells lies parallel to the pore of each stoma.
 - (b) The stomata are always associated with small, bicellular hairs.
 - (c) The epidermal cells in the stomatal areas have thin, pitted lateral walls.
 - (d) The outer walls of the epidermal cells are thick and heavily cutinized.
- (3) That some features are common, though not constant, in the tribe:
 - (a) Prominent venation, with secondaries emerging at a wide angle from a conspicuous midrib.
 - (b) The presence of hypodermal layers.
 - (c) The occurrence of stomata in pits or grooves.
 - (d) The development of cuticular papillae on at least one surface.
 - (e) The subdivision of the mesophyll into quadrangular areas by vertically transcurrent plates of sclerenchyma.

While the exact systematic value of some of these characters may be doubtful, it does appear that a combined anatomical and morphological examination of the leaves of this tribe may successfully separate them both from the remainder of the family and from other families.

Tables 1 and 2 show features of leaves of *Banksia* and *Dryandra* which can be observed in cuticular preparations and transverse section. The general symbol “+” refers either to the presence of a particular character or to its agreement with the adjective used at the top of a column; “—” refers to the opposite condition, and “×” to an intermediate state.

In the column referring to the distribution of the stomata, “P” indicates that they are located in pits, “D” in depressions, “G” in grooves, and “S” that they are more or less superficial. There is no absolute dividing line between pits and depressions, although the two classes are usually quite distinct. The

grooves are not actual cavities in the structure of the leaf, but are formed by the conspicuous curving of the margin towards the midrib of very narrow leaves.

The letters designating the type of hairs refer to the classes described in Section IV(c).

The columns referring to the epidermis between the stomatal areas are not filled in when the stomata are in grooves, as these grooves occupy all the lower epidermis, and the stomata are irregularly scattered over the whole surface. When the stomata are superficially placed, the epidermal characters are noted only when the stomata are restricted to definite areas. The epidermal cells of the actual stomatal areas always have thin, pitted walls, and cuticular papillae are never present.

The cells of the epidermis are only considered to be non-uniform when there is a very conspicuous difference in size between the cells — as, for example, in *Banksia baueri*. The number of papillae per cell is only approximate, and is included solely as a measure of their relative frequency.

Under the heading of hypodermal layers, “t” represents thin-walled cells and “f” thick-walled or fibrous ones; the figures indicate the number of layers present.

VII. VICTORIAN TERTIARY BANKSIEAE

Six Tertiary leaf-types which possess positive characters connecting them with the Banksieae have now to be considered. Their shape (three have entire, one serrated, and two lobed margins), venation, and stomatal structure are quite consistent with such a determination. Moreover, cylindrical hair-bases of the type invariably found accompanying the stomata of living Banksieae also occur. In three of them the stomata are restricted to shallow pits; in the other three they are superficially situated.

Having reached satisfaction regarding the tribal affinities of the fossils, the question of a suitable name arose. Should von Ettingshausen (1888) and Deane (1925) be followed and the lobed types be associated with *Dryandra* and those having undivided or serrated leaf-blades with *Banksia*, or should a new genus be created to indicate relationship to the tribe rather than to either genus? In view of the inseparability of leaves of *Banksia* and *Dryandra*, demonstrated above, the latter course seems to be preferable. The authors therefore propose the name *Banksieaeophyllum* for Australian Tertiary leaves, the form and structure of which conforms to those of living members of the Banksieae.

The present distribution favours this decision. *Banksia* is now widely distributed in Australia, where it is represented by forty-one western and ten eastern species. *Dryandra*, on the contrary, is confined to Western Australia. By referring fossil leaves from New South Wales and Victoria to *Dryandra*, von Ettingshausen (1888), and later Deane (1925), implied that this genus had a wider range in Tertiary times. This may have been the case, but their identifications, based as they were on leaf-form alone, are too uncertain to warrant such an assumption.

TABLE 1 (Continued)
CUTICULAR AND OTHER FEATURES OF *BANKSIA* LEAVES

	LOWER EPIDERMIS										UPPER EPIDERMIS									
	Margins of Stomatal Areas					MIDRIB					Hair Bases					Plates of Sclerenchyma				
	Distribution	Raised	Thickened	Epidermal Outgrowths	Type of Hairs	Cuticle Granular	Cells Uniform	Lateral Walls Pitted	No. Papillae/Cell	Present	Large	No. Cells/H.B.	Lateral Walls Pitted	Hair Bases	Papillae	Present	Large	No. Cells/H.B.	Radial Cells around H.B.	Hy podermal Layers
<i>BANKSIA</i>																				
Sect. 4. <i>Orthostylis</i>																				
<i>B. robur</i> Cav.	P	+	—	—	<i>a,b,d,e</i>	+	+	—	—	+	+	2-4	+	—	—	+	+	1-5	—	1t, 1f
var. <i>minor</i>	P	+	—	—	<i>a,b,e</i>	+	+	—	—	+	+	1-2	+	—	—	+	+	2-5	—	1t, 1f
<i>B. serratifolia</i> Linn.f.	P	+	+	—	<i>a,f</i>	+	+	—	—	+	+	1-3	+	—	—	+	+	1-4	—	1t
<i>B. serratifolia</i> Salisb.	P	+	—	—	<i>a,b,f</i>	+	+	—	—	+	+	1-4	+	—	—	+	+	1-4	—	1t
<i>B. ornata</i> F.v.M.	P	+	—	—	<i>a,b,f</i>	+	+	—	—	+	+	1-3	+	—	—	+	+	1-2	—	1t
<i>B. coccinea</i> R.Br.	P	+	—	—	<i>a,b,f</i>	+	+	—	—	+	+	1-3	+	—	—	+	+	1-3	—	1t
<i>B. sceptrum</i> Meissn.	P	+	—	—	<i>a,f</i>	+	+	—	1-10	+	+	1	+	—	—	+	+	1-2	—	1t
<i>B. menziesii</i> R.Br.	P	+	—	—	<i>a,b,f</i>	+	+	—	1-6	+	+	1	+	—	—	+	+	1-2	—	2t
<i>B. laevigata</i> Meissn.	P	+	—	—	<i>b,e,f</i>	+	+	—	—	+	+	1	+	—	—	+	+	1-8	—	1t
<i>B. hookeriana</i> Meissn.	P	+	—	—	<i>a,f</i>	+	+	—	1-10	+	+	1	+	—	—	+	+	1	—	1t
<i>B. prionotes</i> Lind.	P	+	—	—	<i>a,b,e,f</i>	+	+	—	—	+	+	1-2	+	—	—	+	+	1-4	—	1t
<i>B. victorata</i> Meissn.	P	+	—	—	<i>a,b,f</i>	+	+	—	1-10	+	+	1-2	+	—	—	+	+	1-2	—	1t
<i>B. speciosa</i> R.Br.	P	+	+	—	<i>a,b,f</i>	+	+	—	1-10	+	+	1	+	—	—	+	+	1-4	—	2t
<i>B. baxteri</i> R.Br.	P	+	—	—	<i>b,e</i>	+	+	—	5-20	+	+	1	+	—	—	+	+	1-4	—	1t
<i>B. marcescens</i> R.Br.	P	+	—	—	<i>a,e,f</i>	+	+	—	—	+	+	1	+	—	—	+	+	1-2	—	1t
<i>B. lemanniana</i> Meissn.	P	+	—	—	<i>b</i>	+	+	—	—	+	+	1-4	+	—	—	+	+	1-4	—	1t
<i>B. caleyi</i> R.Br.	P	+	—	—	<i>a,f</i>	+	+	—	—	+	+	1-4	+	—	—	+	+	1-2	—	1t
<i>B. lindleyana</i> Meissn.	P	+	—	—	<i>a,b,f</i>	+	+	—	—	+	+	1	+	—	—	+	+	1-2	—	1t
<i>B. elegans</i> Meissn.	P	+	—	—	<i>a</i>	+	+	—	—	+	+	1-2	+	—	—	+	+	1-2	—	1t
<i>B. candolleana</i> Meissn.	P	+	—	—	<i>b,f</i>	+	+	—	—	+	+	1	+	—	—	+	+	1-4	—	2t
Sect. 5. <i>Isostylis</i>																				
<i>B. ilicifolia</i> R.Br.	P	+	—	—	<i>a,f</i>	+	+	—	—	—	—	—	+	—	—	—	—	—	—	2t
<i>B. ashbyi</i> Bak.f.	P	+	—	—	<i>a,f</i>	+	+	—	—	—	—	1	—	—	—	—	—	1-4	—	2t
<i>B. audax</i> C. A. Gard.	P	+	—	—	<i>b,f</i>	+	+	—	—	—	—	1-2	—	—	—	—	—	1-5	—	1t
<i>B. benthamiana</i> C.A.G. (M.S.)	P	+	—	—	<i>b,f</i>	+	+	—	—	—	—	1	—	—	—	—	—	1	—	1t
<i>B. burdettii</i> Bak.f.	P	+	—	—	<i>a,b,f</i>	+	+	—	—	—	—	1	—	—	—	—	—	—	—	1t
<i>B. drummondiana</i> C.A.G. (M.S.)	P	+	—	—	<i>a,b,f</i>	+	+	—	—	—	—	1	—	—	—	—	—	1	—	1t
<i>B. elderiana</i> F.v.M.	P	+	—	—	<i>a,b</i>	+	+	—	—	—	—	1	—	—	—	—	—	1	—	1t
<i>B. paludosa</i> R.Br.	P	+	—	—	<i>a,e</i>	+	+	—	1-5	—	—	1	—	—	—	—	—	1	—	1t, 1f

TABLE 2 (Continued)

	LOWER EPIDERMIS						MIDRIB				UPPER EPIDERMIS											
Stomata Areas	Margins of Stomatal			Epidermis between Stomatatal Areas			Lateral Walls Pitted	Hair Bases	Papillae	Cuticular Ridges	Epidermal Outgrowths	Cells Uniform	Cuticle Granular	Lateral Walls Pitted	Present	Hair Bases			Radial Cells around H.B.	Hypodermal Layers	Plates of Sclerenchyma	
	Distribution	Thickened	Epidermal Outgrowths	Type of Hairs	Cuticle Granular	Lateral Walls Pitted										No. Papillae/Cell	Large	No. Cells H.B.				Large
Ser. 6. Obvallatae																						
<i>D. sclerophylla</i> Meissn.	D	-	-	a,b,e	+	-	-	-	+	+	+	+	+	+	+	+	+	1-3	-	1t	+	
<i>D. pulchella</i> Meissn.	D	-	-	a,b,e	+	10-20	-	-	+	+	+	+	+	+	+	+	+	1-2	-	1t	+	
<i>D. plumosa</i> R.Br.	D	+	-	a,e,f	+	10-20	X,-	-	+	+	+	+	+	+	+	+	+	1-4	-	1t	+	
<i>D. seneciifolia</i> R.Br.	D	+	-	a,f	+	10-20	X,-	-	+	+	+	+	+	+	+	+	+	1-2	-	1f	+	
<i>D. vestita</i> Kipp.	P	+	+	a,b,e,f	+	1-10	X,-	-	+	+	+	+	+	+	+	+	+	1-4	-	1f	+	
<i>D. circitoides</i> Meissn.	P	+	-	a,e,f	+	1-10	X,-	-	+	+	+	+	+	+	+	+	+	1-2	-	1f	+	
<i>D. hewardiana</i> Meissn.	P	+	-	a,b,f	+	1-30	-	-	+	+	+	+	+	+	+	+	+	1	-	1t	+	
<i>D. patens</i> Benth.	P	+	-	a,b,f	+	10-30	-	-	+	+	+	+	+	+	+	+	+	1	-	1t	+	
<i>D. conferta</i> Benth.	P	+	-	a,b	+	-	X,-	-	+	+	+	+	+	+	+	+	+	1	-	1f	+	
<i>D. horrida</i> Meissn.	P	+	-	a,f	+	1-10	X,-	-	+	+	+	+	+	+	+	+	+	1-2	-	1f	+	
<i>D. serratuloides</i> Meissn.	P	+	-	a,b,e	+	1-10	X,-	-	+	+	+	+	+	+	+	+	+	1-2	-	1f	+	
<i>D. comosa</i> Meissn.	P	+	-	a,b,f	+	1-10	-	-	+	+	+	+	+	+	+	+	+	1-2	-	1f	+	
Ser. 7. Gymnocephalae																						
<i>D. shuttleworthiana</i> Meissn.	P	+	-	a,e	-	1-15	-	-	+	+	+	+	+	+	+	+	+	1	-	1f	+	
<i>D. speciosa</i> Meissn.	P	+	-	a,f	+	2-8	-	-	+	+	+	+	+	+	+	+	+	1-2	-	1f	+	
<i>D. tridentata</i> Meissn.	P	+	-	a,e	+	-	-	-	+	+	+	+	+	+	+	+	+	1-4	-	1t	+	
Sect. 2. Aphragmia																						
<i>D. tenuifolia</i> R.Br.	D	+	-	a,e	-	1-10	-	-	+	+	+	+	+	+	+	+	+	1	-	1t	+	
<i>D. proteoides</i> Lindl.	P	+	-	a,b,f	+	1-20	-	-	+	+	+	+	+	+	+	+	+	1-2	-	1t	+	
<i>D. runcinata</i> Meissn.	P	+	-	a,b,f	+	10-30	-	-	+	+	+	+	+	+	+	+	+	1-2	-	1t	+	
<i>D. obtusa</i> R.Br.	P	+	-	a,b,e	+	1-15	-	-	+	+	+	+	+	+	+	+	+	1-2	-	1t	+	
<i>D. bipinnatifida</i> R.Br.	D	+	-	a,b,e	+	-	+	-	+	+	+	+	+	+	+	+	+	1-2	-	1f	+	
<i>D. pteridifolia</i> R.Br.	P	+	-	a,b,f	+	1-10	X,-	-	+	+	+	+	+	+	+	+	+	1	-	1t, 1f	+	
<i>D. calophylla</i> R.Br.	P	+	-	a,b,e,f	+	1-30	-	-	+	+	+	+	+	+	+	+	+	1-2	-	1t	+	
<i>D. ashbyi</i> Burt	D	+	-	b,d,e	-	1-15	-	-	+	+	+	+	+	+	+	+	+	1	-	1f	+	
<i>D. erythrocephala</i> C.A.Card.	P	+	-	a,b,f	+	1-15	-	-	+	+	+	+	+	+	+	+	+	1-4	-	1f	+	
<i>D. gilbertii</i> S. Moore	P	+	-	a,b,f	+	1-10	X,-	-	+	+	+	+	+	+	+	+	+	1-4	-	1f	+	
<i>D. purdieana</i> Diels	P	+	-	a,b,f	+	1-10	-	-	+	+	+	+	+	+	+	+	+	1-2	-	1f	+	

VIII. GENUS *BANKSIEAEPHYLLUM* n. gen.

Table 3

Leaves simple with entire, serrate, or lobed margins, or pinnate; bilateral. Stomata either superficial, or in pits or grooves, each with a pair of subsidiary cells placed parallel to the pore. Cells of the stomatiferous epidermis with thin, pitted, straight or slightly curved lateral walls, some with bicellular hairs. The basal cells of the hairs are either thick- or thin-walled, of even diameter throughout or expanded distally. Cylindrical hair-bases present on some or all cells of the non-stomatiferous epidermis. Cuticular papillae sometimes present.

BANKSIEAEPHYLLUM *ANGUSTUM* n. sp.

Plate 1, Figs. 1-10

(a) *External Characters*

The description of this type has been derived from a large number of leaf-fragments amongst which, however, neither a complete leaf nor a leaf-apex has been seen, and only exceptionally is the extended petiolar region preserved. The shape of the leaf of *B. angustum* is best shown by the large specimen illustrated in Plate 1, Figure 1. It is narrow-linear and gradually narrows towards both apex and base. The total length preserved in this example is 15 cm. but, allowing for the missing portions, its total length must have approached 20 cm.

The lamina is divided about half-way to the midrib into numerous small, triangular lobes, each with a prominent, forwardly directed mucro (Plate 1, Fig. 3). These segments are frequently opposite but may alternate slightly on either side of the midrib. The leaves, in their central region, are 3-4 mm. wide, the lobes themselves having a depth of about 0.75 mm. Towards the petiole the lobing becomes decreasingly evident until it is represented only by slight crenulations on either side of the midrib (Plate 1, Fig. 4). The margins are strongly revolute.

The midrib is a conspicuous feature of the under surface and has a width of 0.75-1 mm. (Plate 1, Fig. 3). The secondary veins are prominent and arise from the midrib at right angles. One secondary vein terminates in the apex of each lobe and one at each sinus (Plate 1, Fig. 5), two slightly less prominent veins being situated between them. Anastomoses occur between the finer secondary veins, with the result that the lower surface under a low magnification is clearly areolate (Plate 1, Fig. 5).

(b) *Cuticular Structure*

(i) *Lower Epidermis* (Plate 1, Fig. 10).—The stomata occur in rather large shallow depressions which occupy the meshes of the vascular network. These areas measure 120-470 μ across and are separated from one another by the several rows of parenchyma which cover the veins. The stomata are raised above the level of the surrounding epidermis and each is accompanied by a pair of subsidiary cells placed parallel to the pore. The epidermal cells are

TABLE 3
CUTICULAR AND OTHER FEATURES OF *BANKSIAEPHYLLUM* LEAVES

BANKSIAEPHYLLUM	LOWER EPIDERMIS										MIDRIB				UPPER EPIDERMIS										Plates of Sclerenchyma							
	Stomata Areas			Margins of Stomatal			Epidermis between Stomatal Areas							Hair Bases				Papillae	Lateral Walls Pitted	Hair Bases	Cuticular Ridges	Epidermal Outgrowths	Cells Uniform	Cuticle Granular		Lateral Walls Pitted	Papillae	Present	Large	No. Cells/H.B.	Radial Cells around H.B.	Hypodermal Layers
	Distribution	Raised	Thickened	Epidermal Outgrowths	Type of Hairs	Cuticle Granular	Cells Uniform	Lateral Walls Pitted	No. Papillae/Cell	Present	Large	No. Cells/H.B.																				
<i>B. angustum</i>	P	+	—	—	a.f	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>B. acuminatum</i>	P	+	—	—	a.e	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>B. laeve</i>	D	+	—	—	a.?	+	+	+	—	+	+	+	1-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>B. obovatum</i>	S	+	—	—	?	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>B. pinnatum</i>	S	+	—	—	?	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>B. fastigiatum</i>	D	+	—	—	?	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

small, with diameters of about 14-28 μ and vary considerably in shape; their lateral walls are thin, 1.5 μ , straight to slightly curved, and rather strongly pitted. At least 90 per cent. of the cells support thin-walled cylindrical hair-bases. These have basal diameters of about 6-11 μ with either a uniform diameter throughout (cf. type (a)) or are gradually expanded distally as in type (f) of the Banksieae.

(ii) *Midrib*.—The cells are quadrangular and their lateral walls thick and pitted. Hair-bases 11-14 μ wide are numerous near the edges.

(iii) *Upper Epidermis* (Plate 1, Fig. 9).—The cells form a uniform parenchyma; they are small, 16-28 μ in diameter, five- to six-sided, and without hair-bases. Their lateral walls are straight or curved, about 3 μ thick, and unpitted. The cuticle is granular. Hair-bases are present only on the elongated cells which cover the midrib.

(c) *Internal Anatomy*

Plate 1, Figs. 7, 8

The cells of the upper epidermis, as seen in transverse section, are small and cubical; their outer walls, which are about 14 μ thick, are highly cutinized, the cutinization extending into the lateral walls. Between the epidermis and palisade parenchyma are two layers of hypodermis, the cells of which are larger than those of the epidermis. The mesophyll is crossed at intervals by vertical plates of fibres which, as in living Banksieae, pass from the vascular bundles to the hypodermis on either surface. The outer walls of the lower epidermal cells are about 8 μ thick.

Occurrence: Brown coal, Yallourn and Yallourn North, Victoria.

Localities: Shovel face, east side, Yallourn open cut, near pole 70, 9.xi.49. No. 2 coal face, east side, below pole 70, 24.xi.49.

BANKSIEAEPHYLLUM ACUMINATUM n. sp.

Plate 2, Figs. 11-19

(a) *External Characters*

The specimens of *B. acuminatum* do not include a complete leaf, but the preservation in some of the fragments of either the basal or terminal regions allows a fairly accurate picture to be reconstructed. The leaves are broadly linear and taper gradually towards the base and sharply towards the short, pointed apex. The basal region was apparently long, the example shown in Plate 2, Figure 13, measuring 4.5 cm. without showing any appreciable expansion of the lamina. The width of the middle region ranges from 5 to 9 mm. and portions with straight sides of up to 5 cm. are preserved. It seems probable, therefore, that some of the leaves of this species were considerably longer than 10 cm. The leaf margin is entire and strongly recurved.

The midrib, 1-2 mm. wide, and the vascular network are both strongly marked on the under surface (Plate 2, Fig. 14). The secondary veins arise

from the midrib at a wide angle and run out parallel to one another. They are not equally prominent; the stronger laterals 1.5-2 mm. apart bifurcate a short distance from the margin, the branches curving back to join those from the weaker and shorter laterals on either side.

(b) *Cuticular Structure*

(i) *Lower Epidermis* (Plate 2, Figs. 15, 18, 19).—The raised stomata and their accompanying subsidiary cells placed parallel to the pore are restricted to the parenchyma lining rather large pits. The diameter of these pits ranges from 380 to 500 μ . The epidermal cells are small, four- to six-sided, and have diameters of approximately 14-28 μ ; their lateral walls are thin, about 1.5 μ , straight or slightly curved and strongly pitted. Practically all cells, both inside and outside the pits, support hair-bases. These are from 8.5 to 11 μ wide, and are moderately thick-walled. Some hairs have straight sides, and are similar to type (a); others, resembling type (e), have a distal expansion of up to 28 μ with the hair-cell occupying the central portion of the hair-base. The short lengths of the hair-cells retained in some cases suggest that they were probably about 5-6 μ wide (Plate 2, Fig. 19). A few larger hair-bases, 16-22 μ wide, occur on the larger secondary veins.

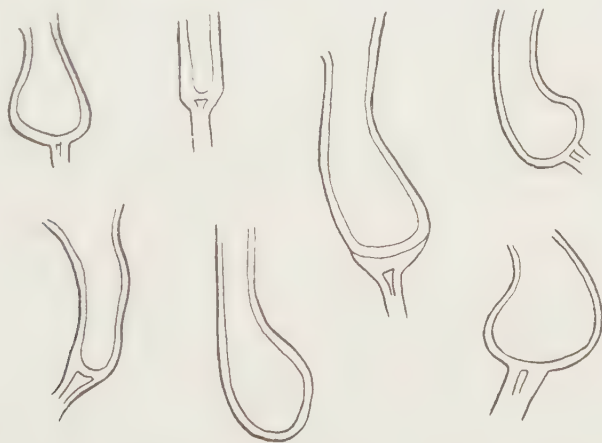


Fig. 4.—Hairs of *Banksiaephyllum acuminatum*. $\times 535$.

(ii) *Midrib*.—The cells covering the midrib are square to quadrangular, their walls are thin and the cross-walls pitted. Short, thin-walled hair-bases 24-25 μ wide are numerous at the edges but sparsely developed or absent from the central region.

(iii) *Upper Epidermis* (Plate 2, Fig. 17).—The parenchyma is uniformly composed of four- to six-sided cells with maximum diameters of 16-36 μ . Their lateral walls are occasionally heavily thickened, 4-8.5 μ wide, straight, and pitted. Large hair-bases are sometimes sparingly present. The cuticle is granular.

(c) Internal Anatomy

Plate 2, Fig. 16

The upper epidermal cells are cubical with much thickened ($16\ \mu$), highly cutinized outer walls. They are separated from the palisade parenchyma by a two-layered hypodermis, the cells of which are larger than those of the epidermis. Vertical plates of fibres occur at frequent intervals in the palisade tissue. The cells of the lower epidermis have cutinized outer walls about $8\ \mu$ thick.

Occurrence: Brown coal, probably from S.E.C. open cut, Yallourn North.

BANKSIEAEPHYLLUM LAEVE n. sp.

Plate 3, Figs. 20-26

The reference of the fragmentary remains of this type to a distinct species of *Banksieaephyllum* is substantiated by their perfectly preserved cuticles. The specific name has been chosen on account of the smooth under surface, due to the inconspicuousness of the veins, a feature in which this form differs from both the previous species.

(a) External Characters

No information regarding the shape of the complete leaf is available but one fragment (Plate 3, Fig. 21) shows that the width of the blade was not uniform. The largest fragment is 4 mm. wide and 1.6 cm. long. The margin is entire, slightly thickened, but not recurved as in *B. angustum* and *B. acuminatum*. A wide midrib (1 mm.) is conspicuous underneath; the fine secondaries, visible on upper surface only, arise from the midrib at a wide angle and run parallel to one another towards the margins of the leaf.

(b) Cuticular Structure

(i) *Lower Epidermis.*—The raised stomata are numerous, superficially situated, and each is accompanied by two rather large subsidiary cells placed parallel to the pore. The interstomatal parenchyma is composed of small cells with thin ($1\ \mu$), straight or somewhat curved, pitted walls and about 90 per cent. support hair-bases. These are rather thin-walled and of two sizes, small ones with straight sides and diameters of $5\text{--}8.5\ \mu$ (cf. type (a)) and large ones $14\text{--}28\ \mu$ across, which are associated with from one to four epidermal cells.

The position of the veinlets is indicated by one to three rows of cells with long axes parallel to the course of the veins; most of these cells carry hair-bases similar to those of the interstomatal parenchyma.

(ii) *Midrib.*—The cells are square to quadrangular and thick-walled, the cross-walls being pitted; hair-bases are present.

(iii) *Upper Epidermis.*—The parenchyma is uniform; its cells are small (Plate 3, Fig. 26), five- to six-sided with straight, unpitted, lateral walls about $2\ \mu$ thick. The cuticle appears to be indistinctly papillate. Hair-bases are absent.

(c) *Internal Structure*

Transverse sections (Plate 3, Fig. 24) show that this leaf was much thinner (approximately 140 μ in the fossilized condition) than leaves of *B. angustum* and *B. acuminatum*. This is largely due to the development of only one layer of small palisade cells and the absence of hypodermal layers. The upper epidermal cells are small, cubical and the outer walls, though cutinized, are relatively thin (3 μ). Slender vertical strands of fibres cross the mesophyll at intervals.

Occurrence: Yallourn, Victoria.

Locality: Open cut, No. 1 batter, east side.

BANKSIEAEPHYLLUM OBOVATUM n. sp.

Plate 4, Figs. 27-34

(a) *External Characters*

These have been derived from the sole specimen available, shown at natural size in Plate 4, Figure 27. The leaf, which is incomplete, measures 7.2 cm. and its maximum width is 12 mm. It is obovate-oblong with an entire, slightly recurved margin and it narrows towards the base. The midrib is relatively broad (1 mm.) on the under surface but the secondary veins are not visible; their position and general direction, however, are indicated on the upper surface by delicate grooves which run from the midrib at right angles.

(b) *Cuticular Structure*

(i) *Lower Epidermis* (Plate 4, Figs. 30-33).—The stomata are superficially situated in clearly defined areas marked out by the several layers of parenchyma cells which cover the veinlets. The guard-cells, which are slightly raised, are accompanied by a pair of inconspicuous subsidiary cells lying parallel to the pore, and the whole stomatal apparatus is surrounded by three or four encircling cells which are smaller and stain more deeply than the general epidermal cells.

The cells of the epidermal parenchyma are of two conspicuously distinct forms, firstly very small, three-, four-, or five-sided cells 5-8 μ in diameter which support thick-walled cylindrical hair-bases of about the same width, and secondly, medium-sized cells of various shapes without hair-bases which have thin, unpitted, curved lateral walls. The cuticle of most of the epidermal cells, including that of the encircling cells of the stomata, is papillate. There are approximately 10 to 20 small papillae per cell (Plate 4, Fig. 31).

(ii) *Upper Epidermis* (Plate 4, Fig. 29).—The cells above the veins are smaller and slightly thicker-walled than those of the areas enclosed by them. All have straight, unpitted walls. Neither hair-bases nor cuticular papillae occur.

(c) *Internal Anatomy*

Plate 4, Fig. 34

The upper epidermal cells are cubical with relatively thin, but highly cutinized, outer walls (3 μ). Hypodermal layers are absent but a well-defined sclerenchymatous network is present.

Occurrence: Brown coal, Yallourn, Victoria.

Locality: Yallourn open cut, No 1 coal face, east side, below pole 30, 9.xi.49.

BANKSIEAEPHYLLUM PINNATUM n. sp.

Plate 5, Figs. 37-42

(a) *External Characters*

Plate 5, Figs. 35, 37

As the sole specimen available was a portion of a leaf about 2 cm. long, no conclusions regarding size or shape can be drawn. The leaf is divided to the midrib into flat, entire, broadly-falcate, obtuse segments 3-4 mm. long and 4-5 mm. broad.

The midrib is very narrow above, 1 mm. broad below. The secondary veins are at right angles to the midrib and run directly to the margin, the most conspicuous being those passing to the tips of the segments; they are seen only on the upper surface.

(b) *Cuticular Structure*

(i) *Lower Epidermis* (Plate 5, Figs. 40, 42).—The stomata occur in slightly depressed areas delimited by the ultimate branches of the veins. They are accompanied by pairs of subsidiary cells lying parallel to the pore and are raised above the surrounding epidermis. The subsidiary cells in this type, however, are often difficult to see and are never prominent.

The epidermal parenchyma is composed of cells of two sizes, moderately large cells with exceedingly thin ($1\ \mu$), straight, pitted walls, and very small cells ($5.5\ \mu$) situated at the junctions of several of the large ones, which support thick-walled cylindrical hair-bases with diameters approximately equal to their own.

(ii) *Midrib*.—The cells are somewhat elongated, and have thin, slightly pitted walls. Small hair-bases are present at the junctions of some of the cells.

(iii) *Upper Epidermis* (Plate 5, Fig. 39).—The cuticle of the upper surface is divided into approximately equal areas by small, thick-walled cells which lie above the veins. The cells become larger and their walls thinner towards the centres of these areas. The lateral walls are unpitted. Hair-bases are absent.

(c) *Internal Anatomy*

Plate 5, Fig. 41

In transverse section the cells of the upper epidermis above the veins appear radially elongated and convergent towards the strands of fibres which cross the mesophyll at rather frequent intervals. The epidermal cells between the veins are square in section. All have cutinized outer walls about $11\ \mu$ thick. The outer walls of the under epidermis are thinner ($5.5\ \mu$). A hypodermis is not developed.

Occurrence: Open cut, Yallourn, Victoria.

BANKSIEAEPHYLLUM FASTIGATUM (Deane) n. comb.

Plate 6, Figs. 43-47

The leaf-type from the brown coal deposit at Yallourn North described by Deane (loc. cit.) under the name *Banksia fastigata* was recently collected at the open cut at Yallourn. This new material, from which the specimen shown in Plate 6, Figure 44, was obtained, agrees so closely with Deane's description and figures of *B. fastigata* that there is little doubt of its identity with that species. Satisfactory cuticular preparations were obtained from it, the study of which has led to the conclusion that *B. fastigata* Deane is allied to the species of *Banksiaephyllum* described above. Since the use of the generic name *Banksia* for fossil types has been shown to be misleading, the authors suggest as a suitable alternative the combination *Banksiaephyllum fastigatum*.

(a) *External Characters*

Plate 6, Figs. 43, 44

The following is Deane's specific description. "Leaves rather long and tapering both at base and apex. The name is given as descriptive of this character. Width, about $3/5$ inch, regularly toothed, the lobes turned towards the apex, sinuses often one-third of the distance from midrib to tip of tooth, lateral veins curved, one terminating each tooth and one each sinus."

(b) *Cuticular Structure*

(i) *Lower Epidermis* (Plate 6, Figs. 47, 48).—The stomata are situated in slight depressions, the long axes of which tend to be oriented in one direction. The individual stomata appear to be slightly sunk below the surface of the surrounding epidermis. Lying parallel to the pore of each stoma are two inconspicuous subsidiary cells, over which the neighbouring epidermal cells may project slightly. The stomatal apparatus is surrounded by from three to six narrow cells which stain deeply.

The epidermal parenchyma is not uniform. Most of its cells are of medium size with, in the stomatal areas, straight or curved, very thin and pitted walls, and above the veins similar but somewhat thicker walls. Amongst them are scattered exceptionally small cells, 4-6 μ in diameter, which support hair-bases of similar size.

(ii) *Midrib*.—The cells are small, square to quadrangular, with moderately thick, unpitted walls; small hair-bases are sparsely developed. The cuticle is longitudinally ridged.

(iii) *Upper Epidermis*.—The cells above the veins are somewhat smaller than those of the areas enclosed by them. All have rather thin, unpitted, straight or slightly curved walls.

Occurrence: Brown coal, Yallourn and Yallourn North, Victoria.

IX. DISCUSSION

The six species of *Banksieaephyllum*, while agreeing in general form and structure with living members of the Banksieae, cannot be identified with any particular species of this tribe.

In view of the present restriction to Western Australia of species of *Banksia* having pinnate leaves or simple leaves with deeply lobed margins, it is of interest to note the occurrence in eastern Australia of fossil Banksieae possessing these features. In *Banksieaephyllum angustum* the lamina is divided about half-way to the midrib into triangular segments, while in the leaf of *B. pinnatum* the subdivision extends to the midrib. The fossil leaf of *Dryandra urniformis* Deane from Yallourn North is also pinnate but since the authors have been unable to examine the structure of this species further comment is not made.

Deane (1925, p. 494) has already referred to the broad apex typical of leaves of living species and contrasted it with the tapering tip of his species *B. fastigata* (here referred to as *Banksieaephyllum fastigatum*). The leaf of *B. acuminatum* also has an acute apex. The apex of *B. obovatum*, on the other hand, is obtuse and the general form of the leaf closely approaches that of the Victorian species *Banksia integrifolia*.

The leaves of the species of *Banksieaephyllum* show the same variations in the disposition of the stomata as those of living Banksieae. In *B. acuminatum* and *B. angustum* the stomata line the epidermis of distinct pits. In *B. pinnatum*, *B. obovatum*, and *B. fastigatum* they are superficially placed in distinct areas marked out by the cells covering the veins. In *B. laeve* they are diffusely arranged as in *Dryandra kippistiana*.

When considered collectively, the species of *Banksieaephyllum* appear to fall naturally into two groups according to the character of the parenchyma of the upper and lower surfaces and the degree of hairiness of the latter. The parenchyma of the under surface of *B. angustum*, *B. acuminatum*, and *B. laeve* is uniform and strongly hairy, the hair-bases approximating closely to those of some species of *Banksia* and *Dryandra*. In *B. pinnatum*, *B. obovatum*, and *B. fastigatum*, however, hair-bases are sparsely developed and the parenchyma is not uniform, with a marked difference in size and shape between the small cells which support the hair-bases and those of the larger ones of the general epidermis. The epidermal parenchyma of several species of *Banksia* and *Dryandra* is similarly differentiated, sometimes on the upper as well as the lower surface, e.g. *B. benthamiana* (Plate 8, Fig. 61). They also show the same pronounced difference in size, but in none are the cells with hair-bases as small as they are in the fossil species, where the diameter is only about 5.5 μ .

The upper epidermis of *Banksieaephyllum angustum*, *B. acuminatum*, and *B. laeve* is composed of uniform straight-sided cells with thick lateral walls. Hair-bases, as in a few living Banksieae (Tables 1, 2) are usually entirely absent, but occur, occasionally, in *B. acuminatum*. In *B. obovatum*, *B. pinnatum*, and *B. fastigatum* the upper epidermis has a patterned appearance due to the different size and shape of the cells covering the veins from those of the areas enclosed by them.

The hair-bases of the fossils are, on the whole, thinner-walled than those of the living species, where the walls usually attain a considerable thickness. In only one species—*B. laeve*—are large and small hair-bases associated together as they are, for example, in *Banksia robur*. The occurrence in *B. angustum* and *B. acuminatum* of hair-bases closely similar to those of types (e) and (f) of living forms is of interest.

The development of cuticular papillae in *Banksieaephyllum laeve* and *B. obovatum* provides a close link with living Banksieae, in which they are frequently present. In *B. laeve* the papillae, which are only faintly defined, are restricted to the cuticle of the upper epidermis. In *B. obovatum* they are more clearly outlined and occur on all the cells of the lower epidermis without hair-bases.

As far as the internal structure of the fossil leaves is concerned, that of *B. acuminatum* and *B. angustum* approaches most closely to the structure typical of leaves of *Banksia* and *Dryandra*. The outer walls of the upper epidermal cells are considerably thicker than those of the other fossil species; there is a two-layered hypodermis in both types and the anastomosing sclerenchymatous strands are numerous and prominent. In contrast to this the outer walls of the upper epidermal cells of *B. laeve* and *B. obovatum* are thinner than those of any living species. In both species, as well as in *B. pinnatum*, a hypodermis is not developed. This structure is absent only from the leaves of *Dryandra kippistiana*, *D. serra*, and *D. concinna*.

X. FRUITING CONES OF BANKSIA SP.

Plate 7, Figs. 49-56

Evidence of the presence of *Banksia* in beds regarded by early Victorian geologists as of older Pliocene age was provided by the discovery of fruiting cones and wood during gold mining operations almost a century ago. The age of the Victorian older deep leads is still in doubt but Mr. W. Baragwanath and Dr. D. Thomas of the Victorian Mines Department believe that they are probably pre-Pliocene.

The earliest record of the occurrence of *Banksia* appeared in a paper by Redeway (1858) on the gold diggings of Creswick Creek and Ballarat, in which he remarked "The cones of the 'honeysuckle' or *Banksia* have been found not unfrequently in this drift. These are very brittle, but the wood is often well preserved." In a footnote the author stated that some of the cones "were submitted by Sir R. Murchison to the late Dr. R. Brown, who identified them as belonging to *Banksia*."

Several later references occur in the Reports of Progress of the Geological Survey of Victoria. Smyth in 1873 stated that "In the leads at Chiltern and at Creswick the cones of the *Banksia*, scarcely to be distinguished from those gathered now in the forest, are associated with rich auriferous gravels." In 1875 he wrote further that "Cones of *Banksia* (like *Banksia ericifolia*) are found in some leads."

Since fossil fruiting cones of *Banksia* have not hitherto been illustrated, a photograph has been included (Plate 7, Fig. 49) of a specimen belonging to the Geological Survey Museum from the deep leads at Eldorado, Victoria. It contains a high proportion of mature fruits, in a few of which the valves are widely separated.

Banksia cones occur also in lignite from an unspecified locality at Yallourn, thus confirming other evidence of the presence of the genus in early Tertiary times. Two specimens have been available for examination, and both are figured in Plate 7. The one shown in Plate 7, Figure 50, is a small cone 4.5 cm. long and about 2 cm. wide. On its exposed surface bracts and bracteoles can be clearly distinguished, but no fruits are evident. This cone may have been sparingly fertile or even infertile, a condition often met with in living species.

The second cone has been split longitudinally, and the apical region, shown enlarged in Plate 7, Figure 52, completely detached. Its impression, however, can be seen in the half illustrated in Plate 7, Figure 51. This cone is 5.5 cm. long and 3 cm. wide and its woody axis measures 5 mm. across. A large number of bracts and bracteoles, whose radial measurement is about 2 mm., are crowded on the axis and amongst them are suggestions of possible fruit valves. The bracts can be removed from the axis and preparations made of the matted hairs with which they are invested. These hairs (Plate 7, Fig. 55) are long, about 22 μ wide, and have moderately thick, spirally twisted walls similar to those of type (*d*) of the living *Banksiae* (Fig. 1). Cuticles detached from the bracts show that the basal cells of these hairs are thin-walled and about 22 μ wide throughout. Hairs showing features similar to these occur on bracts of *Banksia integrifolia* and *B. marginata* (Plate 7, Fig. 56).

Occurrence: Deep leads, Eldorado, Victoria. Brown coal, probably from the open cut at Yallourn North, Victoria.

XI. RECORDS OF LEAVES OF THE BANKSIEAE FROM AUSTRALIAN TERTIARY DEPOSITS

In order to complete the account of the fossil *Banksieae*, a list is included of all Australian records which have been validly published (Table 4). Fossil leaves which have been identified as members of this tribe have been found in Tasmania, Victoria, New South Wales, Queensland, South Australia, and Central Australia. Although *Banksia* and *Dryandra* have reached their greatest present-day development in Western Australia, no fossil species has been recorded from that State. This is doubtless partly due to the paucity of Tertiary deposits there, and to the fact that little palaeobotanical research has been carried out in connection with these deposits.

A total of 19 species has been previously recorded. However, much of the material on which these determinations have been based is fragmentary, and thus descriptions and illustrations are frequently inadequate. Without other evidence, it is impossible to decide whether all these species are really

distinct, or to confirm their affinities with the tribe Banksieae or even the family Proteaceae. Because of these difficulties, no attempt has been made to include these fossils in the genus *Banksiacaphyllum* unless type specimens have been available.

TABLE 4
AUSTRALIAN RECORDS OF FOSSIL BANKSIEAE AND MYRICACEAE

Species	Tas.	Vic.	N.S.W.	Qld.	Cent. Aust.
Banksieae					
<i>Banksia adunca</i> Deane (1925)		x			
<i>B. blaxlandi</i> Ett. (1888)			x		
<i>B. campbelli</i> Ett. (1888)			x		
<i>B. crenata</i> Ett. (1894)				x	
<i>B. cretacea</i> Ett. (1894)				x	
<i>B. fastigata</i> Deane (1925)		x			
<i>B. hoveilli</i> Ett. (1888)				x	
<i>B. lancifolia</i> Ett. (1888)	x		x		
<i>B. lawsoni</i> Ett. (1888)			x		
<i>B. myricaefolia</i> Ett. (1888)			x		
<i>B. plagioneura</i> Ett. (1894)				x	
<i>B. poolii</i> Ett. (1888)			x		
<i>B. praegrans</i> Chap. (1937)					x
<i>B. sub-longifolia</i> Ett. (1894)				x	
<i>B. cf. marginata</i> Cav. Chap. (1937)					x
<i>Dryandra benthami</i> Ett. (1888)				x	
<i>D. praeformosa</i> Ett. (1888)				x	
<i>D. urniformis</i> Deane (1925)		x			
<i>Dryandroides johnstonii</i> Ett. (1888)	x				
Myricaceae					
<i>Myrica eyrei</i> Ett. (1888)	x				
<i>M. konincki</i> Ett. (1888)			x		
<i>M. pseudo-lignitum</i> Ett. (1894)				x	
<i>M. pseudo-salix</i> Ett. (1888)			x		
<i>M. subsalicina</i> Shirley (1898)				x	
<i>Myricophyllum longepetiolatum</i> Ett. (1894)				x	

Deane (1900) points out that there is frequently a strong resemblance between the leaves of some *Banksia* and *Dryandra* species and those of certain species of *Myrica*. The similarity between these forms, coupled with the facts of their present-day distribution, tend to suggest that Australian fossil representatives of the genus *Myrica* may have been incorrectly identified. If this is so, it is possible that they should be placed in the tribe Banksieae. Table 4 includes the species of *Myrica* described from Australian Tertiary deposits, although it is impossible to voice an opinion as to their true affinity until further material is available.

XII. PALAEOECOLOGICAL CONSIDERATIONS

The six species of *Banksiaephyllum* described in the present paper exhibit various degrees of xeromorphism, this phenomenon being particularly evident in *B. angustum* and *B. acuminatum*. It is of interest, therefore, to consider the plants with which they may have been associated and the climatic conditions under which they may have lived.

The first published work on fossil plants from the lignites at Yallourn and the Latrobe valley generally appeared in 1922 when Miss Nobes described the wood of four conifers. No definite assertions were made, however, regarding their relationship with living genera.

In 1925, Chapman published two papers dealing with material from the same area. In one he noted wood fragments which he considered were derived from trees allied to *Callitris* and *Casuarina*, in the other a seed resembling those of modern cycads. In the same year Deane described and illustrated leaves which he identified as belonging to species of *Banksia*, *Lomatia*, *Dammara* (now *Agathis*), *Ginkgo*, *Phyllocladus*, *Cinnamomum*, and *Tristania*.

In 1945, Cookson reported the occurrence of pollen grains of the Podocarpaceae, *Casuarina*, and *Nothofagus* in the lignites of eastern Victoria and in 1946 recognized the pollen grains of three distinct species of *Nothofagus* from this Yallourn deposit; a leaf believed to have belonged to an oleaceous plant was described in 1947 (Cookson 1947a).

The association of xeromorphic Banksieae with *Nothofagus* and other rain-forest types appears incompatible until it is realized that a similar association of divergent ecological types exists in the present flora of certain parts of Australia. In the Dave's Creek area of the Lamington Plateau, southern Queensland, for example, *B. serratifolia* occurs in close proximity to a luxuriant rain-forest in which are found conifers such as *Podocarpus* and *Araucaria*, and species of *Cinnamomum*, *Lomatia*, *Tristania*, and *Casuarina*. On Mount Hobwe, only eight miles away, is a forest dominated by *Nothofagus moorei* (F. Muell.) Maiden. Cycads also occur in southern Queensland.

It will be seen, therefore, that species present in the rain-forest areas of warm-temperate Queensland include some that are closely allied to forms identified from the Yallourn deposits. This fact was noticed by Deane, who tentatively inferred that "the general temperature of the southern part of Australia when the brown coal of Morwell was deposited was higher than it is at the present day, that warmth and moisture abounded, and that hot dry winds did not occur, or at least were a rarity." Patton (1933) and Crocker and Wood (1947) have expressed similar opinions.

That the air was moist at this time is also suggested by the discovery of a variety of epiphyllous fungi on leaves preserved in the brown coal (Cookson 1947b).

It is more difficult to deduce the thermal conditions prevailing from the evidence of the plants which existed during this period, particularly as the true identity of these plants is frequently uncertain. In some instances, the problem of distribution is complicated by the influence of ecological factors other than

climate. Also, as Seward (1931) points out, closely related plants often occur in a wide variety of habitats and hence are useless as indicators of climate. It is even possible, as Seward suggests, that plants of the Tertiary period may have tolerated wider ranges of temperature and rainfall than those of the present day.

However, if the distribution of a fossil flora as a whole, rather than that of individual genera and species is considered, these difficulties may be of reduced importance. A number of fossil plants in the Yallourn brown coal have not yet been identified and it is possible that further work may produce more definite evidence regarding the Tertiary climate of south-eastern Victoria.

XIII. RECORDS OF EXTRA-AUSTRALIAN BANKSIEAE

A number of isolated leaves from extra-Australian deposits have been referred to *Banksia* and *Dryandra* (Kausik 1943) but some, at least, of the identifications seem extremely doubtful. Since the authors have seen only Australian material this matter is not discussed further; at the same time, however, they desire to reiterate their belief that misleading conclusions may be drawn when detached leaves are attributed to either genus.

Two examples from the present study may be cited in support of this statement. The Victorian fossil leaf *Banksiaephyllum obovatum* is very similar in shape to that of the living species *Banksia integrifolia* but the cuticular and internal structure of the two species, as can be seen in Tables 1 and 3, are completely different. To mention only one of the distinctions, the stomata of *B. integrifolia* occur in well-defined pits (Plate 8, Fig. 57) whereas those of *B. obovatum* are superficially placed (Plate 4, Fig. 30). A second example is afforded by the fossil leaf *B. pinnatum*, which externally is closely similar to that of *Dryandra formosa*, but structurally is quite distinct from that species. Moreover, there is no reason for associating the fossil with *Dryandra* rather than with *Banksia*. With such facts in mind, it is difficult not to question the authenticity of the assignment by Berry (1916) of an American leaf to *Banksia lennifolia* and the tentative suggestion put forward that it is most like *Banksia spinulosa*, a New South Wales species. As none of the Victorian fossil leaves discussed in this paper can be satisfactorily identified with living Australian species, it seems extremely doubtful whether extra-Australian types, should they exist, would closely approach living Australian forms.

Kirchheimer (1937) expressed doubts as to whether leaves from European deposits referred by Viniklar and other authors to *Banksia* and *Dryandra* are correctly placed and reiterated earlier suggestions that they may have belonged to myricaceous plants.

Professor T. G. Halle (1940) referred briefly to this question and the following paragraph (translated by Dr. Britta Lundblad) presents his considered opinion.

Earlier authors describe a great number of remains supposed to be referable to the Proteaceae from Europe and North America, but their determinations

are probably all misinterpretations, the remains concerned belonging to several different families. Recently, a few data considered to be of a more reliable kind have been added, viz. the report of several species of leaves and a young inflorescence from the Upper Cretaceous of Bohemia, and an infructescence from the Eocene of England. In addition, a species of *Banksia* from the Eocene of southern U.S.A. is considered by Berry to be correctly determined. The question of the early appearance of the Proteaceae in Europe is still under discussion, however.

XIV. *PHYLLITES YALLOURNENSIS* n. sp.

A rather common leaf in the Yallourn seam is the one described below under the name of *Phyllites yallournensis* (Plate 9). It is included in this paper because it shows a superficial likeness to leaves of certain Banksieae and as an illustration of the unreliability of assuming affinity with this tribe on similarity of leaf form alone. Apart from their smaller size, the resemblance of leaves of *P. yallournensis* to those of *Dryandra calophylla* is particularly strong. When, however, the cuticular characters of the two species are compared it becomes evident that they are distinct and unrelated. *P. yallournensis* possesses none of those characteristics which, as has been shown, belong to the Banksieae. Moreover, there seems no reason to think that it belonged to the Proteaceae at all.

The leaves of two extra-Australian plants, both native of North America, whose shape approaches that of the leaves of *P. yallournensis* must be mentioned. They are *Lyonothamus floribundus* Gray var. *asplenifolium* Brand, of which individual pinnae bear comparison, and *Comptonia peregrina* Coulter, where the leaf is simply pinnate. An examination of the cuticles of these species has shown, however, that the structure is quite distinct from that of *P. yallournensis* and suggests that the Australian fossil leaf is unrelated to either. The affinity of *P. yallournensis* thus remains obscure. Although it probably belonged to a dicotyledonous plant, it appears preferable to place it in the form genus *Phyllites* until more information becomes available.

(a) *External Characters*

Plate 9, Figs. 64-6

The specimens, though numerous, are fragmentary, the longest piece being 4 cm. The leaf is pinnate, the segments becoming smaller towards the base. The pinnae are numerous, 2-7 mm. long and 1.5-4 mm. broad, oblong-obtuse or slightly narrowed towards the apex, broadly attached and contiguous at the base, opposite or slightly alternate on either side of the midrib, the margin entire, not revolute. The midrib is relatively wide (about 1 mm.) underneath; the secondary veins are inconspicuous, parallel, arising at a wide angle, and frequently converging towards the apex of the segments.

(b) *Cuticular Structure*

(i) *Lower Epidermis* (Plate 9, Figs. 68, 69).—The stomata are superficial and irregularly oriented in large areas marked out by the cells covering the veins. They are oval to isodiametric, the long axes of the oval ones ranging from 16 to 28 μ ; the poral rim has a narrow cutinized edge, and the dorsal wall is delicate. The stomata are usually surrounded by three or four rather small cells which may be of the nature of subsidiary cells. These are irregular in shape and their position in relation to the guard cells varies considerably.

The interstomatal epidermal cells are small with rather thin, straight or curved, pitted lateral walls. The cells covering the veins have slightly thicker, sparingly pitted walls and their long axes are parallel to the course of the veins. Hair-bases are absent.

(ii) *Midrib*.—Cells small, square, quadrangular or triangular, with straight, moderately thick, pitted walls. Small hair-bases sparsely distributed.

(iii) *Upper Epidermis*.—The cells of the epidermal parenchyma are small, 14-25 μ in diameter, with thin, straight or slightly curved, unpitted lateral walls; those above the veins are smaller and thicker-walled than those situated in the areas they delimit. The cuticle is granular. Hairs are not developed.

(c) *Internal Structure*

The tissues of the leaves available are rather crushed and distorted, but in small areas of transverse sections, such as the one shown in Plate 9, Figure 70, the differentiation of the mesophyll is reasonably clear. The cells of the upper epidermis are square in section and the outer walls, 3 μ thick, are cutinized. There are no hypodermal layers. Beneath the epidermis is a single layer of palisade parenchyma. The under epidermal cells are similar to those of the upper surface. The vertical strands of parenchyma which cross the mesophyll in the Banksieae appear to be absent from the leaves of *P. yallournensis*.

Occurrence: Brown coal, Yallourn, Victoria.

Locality: Yallourn open cut, No. 1 coal face, east side, below pole 30, 9.xi.49.

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EXPLANATION OF PLATES 1-9

The photographs are the work of I. Cookson except those in Figures 28, 37, 44, and 65 which were taken by Mr. E. Matthaei and those in Figures 20, 21, 22, 51, and 69 which were taken by Miss M. Johnson. All figures are from untouched negatives. The letters N.M.V. before a specimen number refer to the collection of the National Museum of Victoria.

PLATE 1

Banksieaephyllum angustum n.sp.

- Fig. 1.—An almost complete leaf 3/5 natural size. (N.M.V. No. 14734.)
 Fig. 2.—Upper surface of a small leaf fragment. Natural size. (N.M.V. No. 14848.)
 Fig. 3.—A small leaf fragment showing prominent venation on the under surface. $\times 4$.
 Fig. 4.—Proximal region with lamina narrowing towards the petiole. Natural size. (N.M.V. No. 14735.)
 Fig. 5.—A piece of cuticle showing the course of the secondary veins and the presence of stomatal pits on the lower surface. $\times 24$.
 Fig. 6.—Cuticle of lower surface with stomatal pits and numerous hair-bases. $\times 50$.
 Fig. 7.—Transverse section of leaf showing revolute margin and subdivision of mesophyll by vertical plates of sclerenchyma. $\times 100$.
 Fig. 8.—Transverse section showing upper epidermis, two-layered hypodermis, and palisade parenchyma. $\times 250$.
 Fig. 9.—Cuticle of upper surface. $\times 300$.
 Fig. 10.—Cuticle of lower surface showing stomata and hair-bases. $\times 300$.

PLATE 2

Banksieaephyllum acuminatum n.sp.

- Fig. 11.—Leaves with acuminate apices viewed from above. Natural size. (N.M.V. No. 14736.)
 Fig. 12.—Portion of a leaf showing narrowing of lamina. Natural size. (N.M.V. No. 14737.)
 Fig. 13.—Proximal portion of a leaf with slender petiole. Natural size. (N.M.V. No. 14737.)
 Fig. 14.—Under surface of leaf to show inrolled margin, wide midrib, and prominent venation. $\times 4\frac{1}{2}$. (N.M.V. No. 14738.)
 Fig. 15.—Cuticle of lower epidermis showing anastomosing veins and stomatal pits. $\times 50$.
 Fig. 16.—Transverse section of leaf showing upper epidermis, two-layered hypodermis, palisade parenchyma, sclerenchyma, lower epidermis. $\times 100$.
 Fig. 17.—Cuticle of upper epidermis; l.h.b., large hair base. $\times 300$.
 Fig. 18.—Cuticle of interstomatal epidermis with stomata and hair-bases. $\times 300$.
 Fig. 19.—Cuticle of interstomatal epidermis showing bicellular hairs. $\times 300$.

PLATE 3

Banksieaephyllum laeve n.sp.

- Fig. 20.—Portion of a leaf from above. $\times 3$. (N.M.V. No. 14739.)
 Fig. 21.—A fragment showing narrowing of lamina. $\times 6$. (N.M.V. No. 14740.)
 Fig. 22.—Leaf from below, showing smooth surface, prominent midrib, and entire, flat margin. $\times 5$. (N.M.V. No. 14741.)
 Fig. 23.—Cuticle of lower epidermis with diffusely arranged stomata. $\times 100$.
 Fig. 24.—Transverse section of leaf showing upper epidermis, palisade parenchyma, under epidermis with hair-bases, and sclerenchymatous strand cut longitudinally. $\times 250$.
 Fig. 25.—Cuticle of lower epidermis showing stomata with subsidiary cells, and hair-bases of two sizes. $\times 350$.
 Fig. 26.—Cuticle of upper epidermis showing cells with cuticular papillae. $\times 300$.

PLATE 4

Banksieaephyllum obovatum n.sp.

- Fig. 27.—Type specimen from above. Natural size. (N.M.V. No. 14741.)
 Fig. 28.—A small portion of the type from below showing wide midrib and smooth surface. $\times 5$.
 Fig. 29.—Cuticle of upper epidermis. $\times 100$.
 Fig. 30.—Cuticle of lower epidermis showing superficial stomata. $\times 100$.
 Fig. 31.—Lower cuticle showing small cells with hair-bases and larger cells with cuticular papillae. $\times 350$.

Fig. 32.—Lower cuticle showing presence of cuticular papillae on encircling cells of stomata. x 550.

Fig. 33.—Lower cuticle, the stoma in the lower right-hand corner focused to show a pair of subsidiary cells lying parallel to the pore.

Fig. 34.—Transverse section of leaf showing two vertical strands of fibres—the one to the right being cut obliquely. x 250.

PLATE 5

Banksiaephyllum pinnatum n.sp.

Figs. 35 and 36.—Type specimen. Natural size and x 3. (This specimen was destroyed to obtain its cuticle.)

Fig. 37.—Portion of the lower surface of the same specimen. x 5.

Fig. 38.—Cuticle of the lower epidermis showing stomatal areas and cells of two sizes. x 100.

Fig. 39.—Cuticle of upper epidermis. x 100.

Fig. 40.—Cuticle of lower epidermis with stomata and their subsidiary cells. x 300.

Fig. 41.—Transverse section of the leaf showing mesophyll crossed by two vertical strands of fibres, and thick outer walls of epidermal cells. x 250.

Fig. 42.—Cuticle of lower epidermis. Small cells with hair-bases can be distinguished from larger ones without hairs; subsidiary cells related to two stomata can be seen in the lower right-hand corner. x 350.

PLATE 6

Banksiaephyllum fastigatum (Deane) n.comb.

Fig. 43.—The specimen of *Banksia fastigata* illustrated by Deane in Plate 61, Figure 4, of his paper (1925). x 2.8.

Fig. 44.—A small leaf-fragment. x 4. (N.M.V. No. 14780.)

Fig. 45.—Cuticle of upper epidermis. x 100.

Fig. 46.—Cuticle of lower epidermis. x 100.

Figs. 47, 48.—Cuticle of lower epidermis showing small cells with hair-bases, stomata with subsidiary cells, and elongate cells covering the veins. x 350.

PLATE 7

Fruiting cones of *Banksia* sp.

Fig. 49.—Fertile cone of *Banksia* from Eldorado, Victoria. Natural size. (Geol. Surv. Mus. No. 50750.)

Fig. 50.—A cone from Yallourn. Natural size. (N.M.V. No. 14742.)

Fig. 51.—Median longitudinal view of a cone from Yallourn, showing central axis, bracts, a possible fruit on left-hand side, and the impression of the apex of the cone. Natural size. (N.M.V. No. 14743.)

Fig. 52.—Surface view of counterpart of apical region of the previous specimen showing bracts and bracteoles. x 4. (N.M.V. No. 14775.)

Fig. 53.—Transverse view of second half of the same specimen showing bracts covered with matted hairs. x 2. (N.M.V. No. 14776.)

Fig. 54.—Cuticle of a bract showing hair-bases. x 300.

Fig. 55.—Hairs from a bract of No. 14776. x 100.

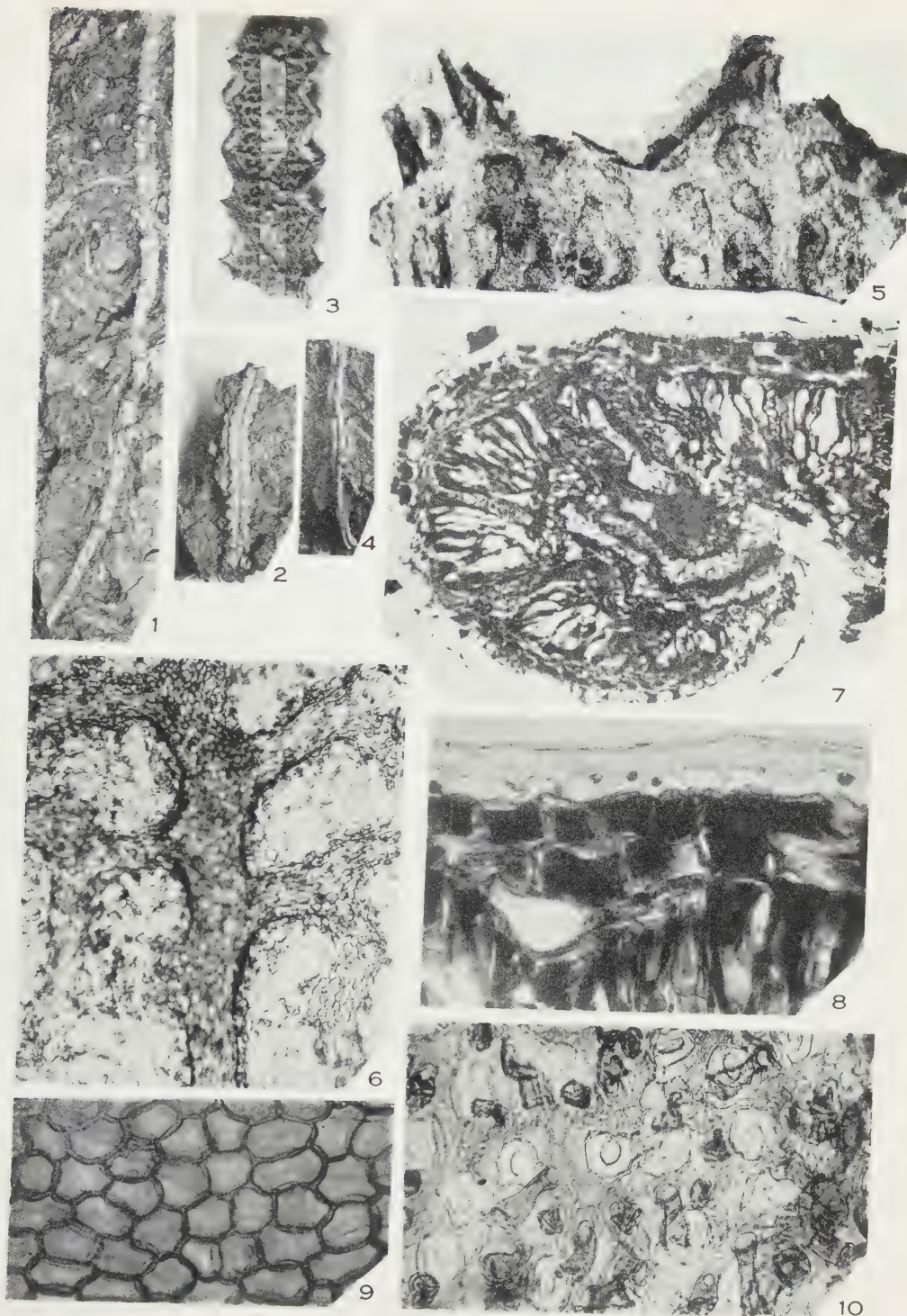
Fig. 56.—A hair from a bract of *Banksia marginata*. x 100.

PLATE 8

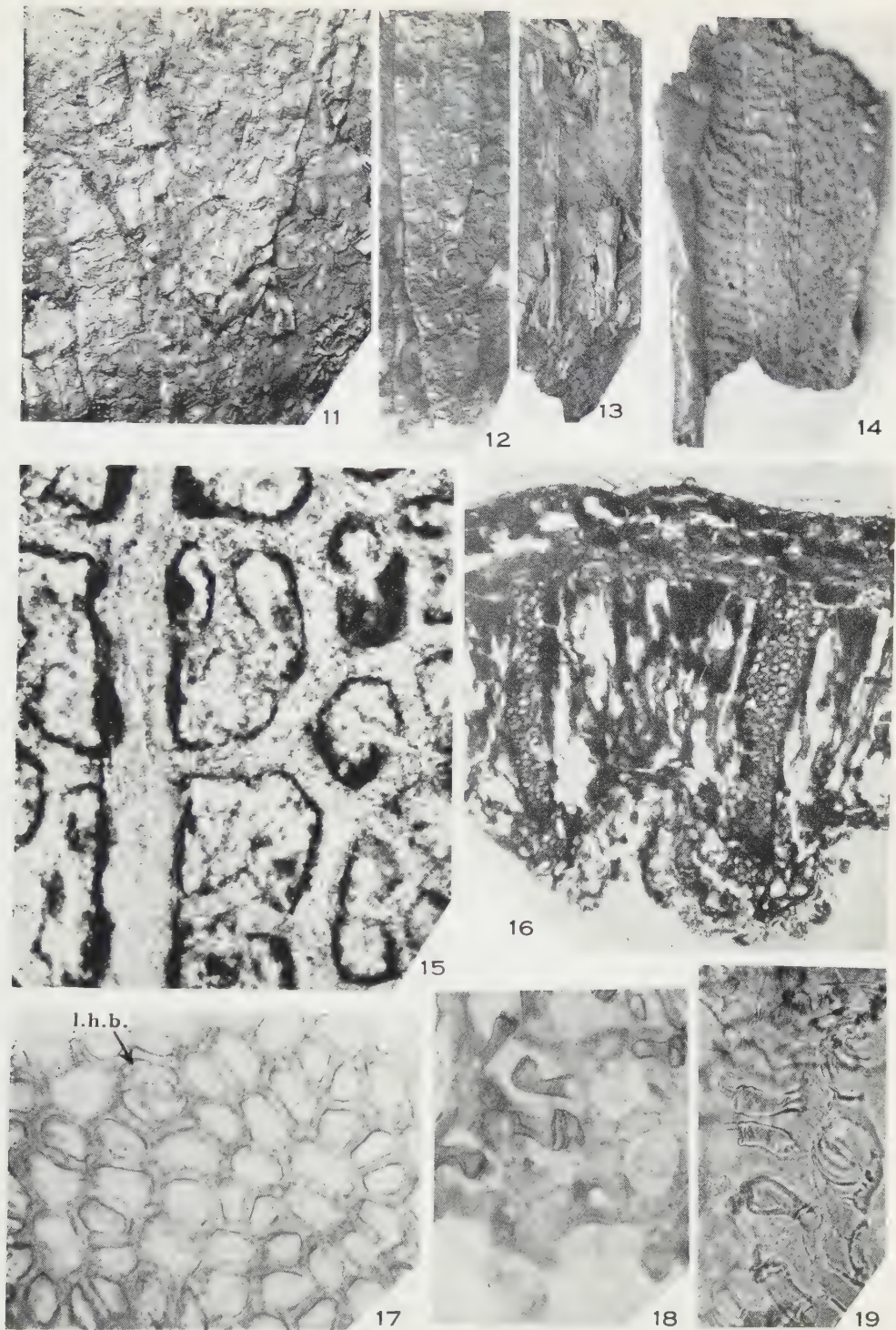
Structural features of living Banksieae

Fig. 57.—Lower cuticle of *Banksia integrifolia* showing stomatal pits and prominent veins. x 40.

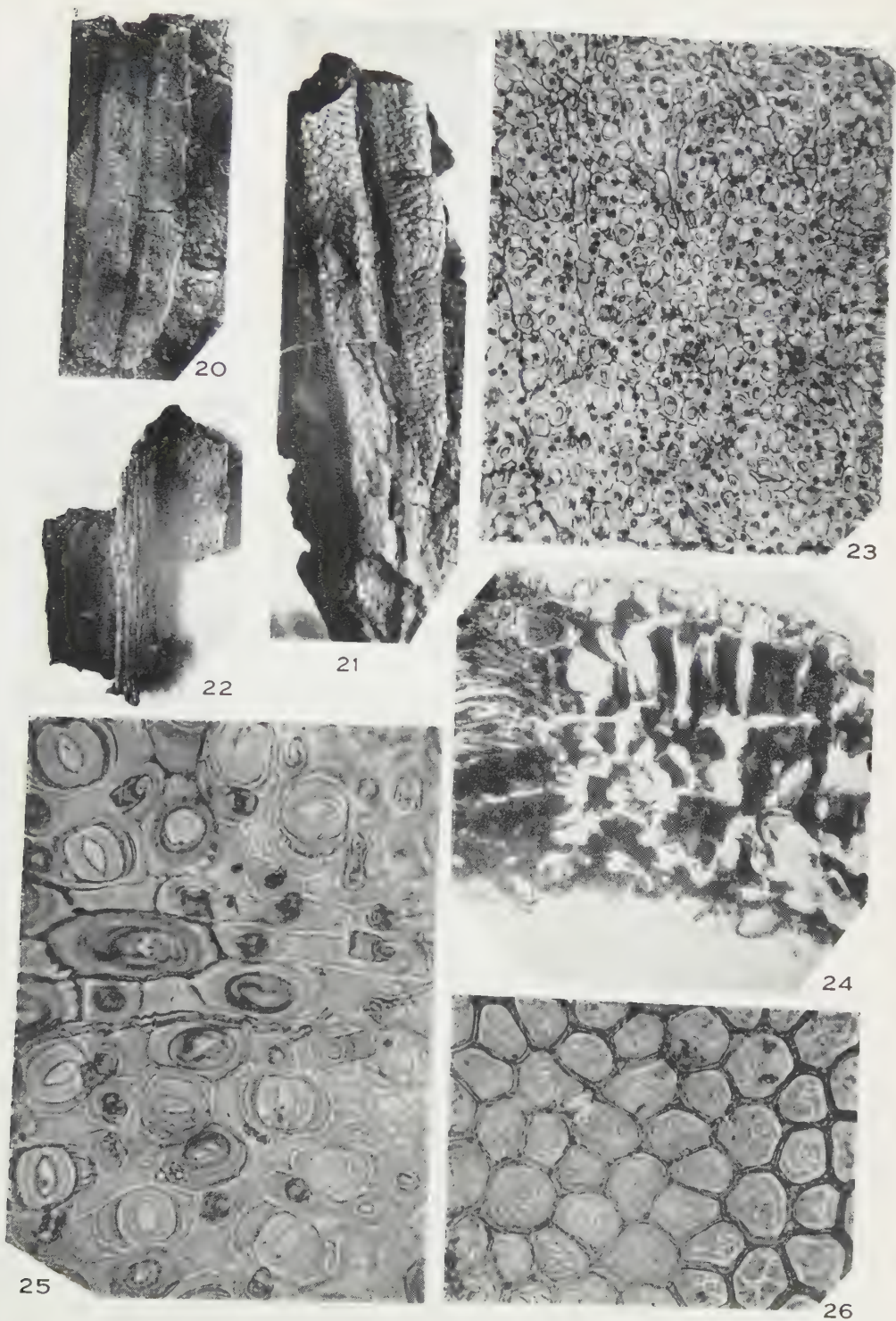
Fig. 58.—Lower cuticle of *Banksia quercifolia* showing epidermal papillae around and near the pits. x 100.



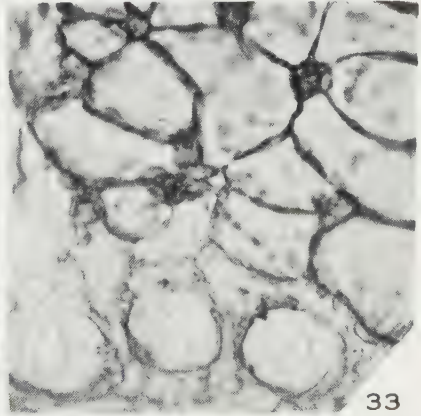
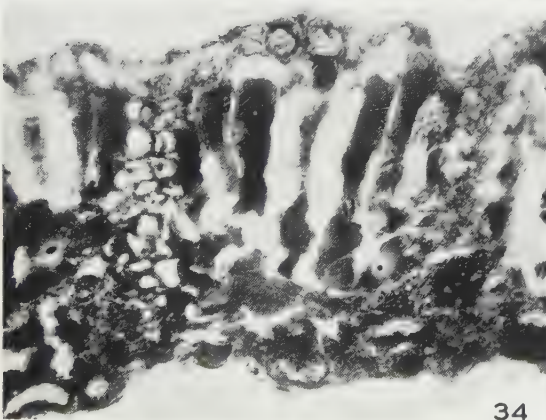
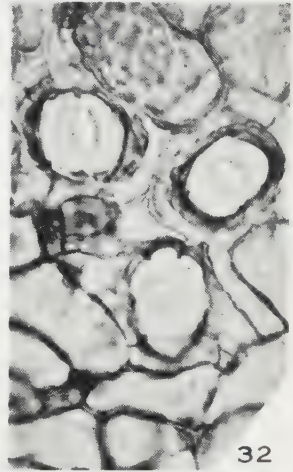
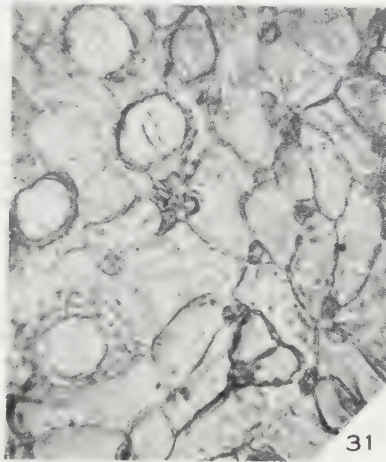
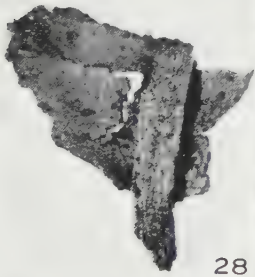
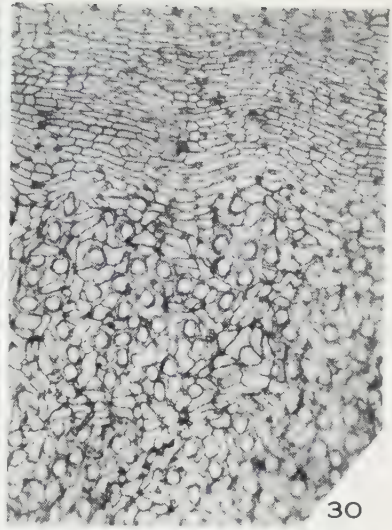
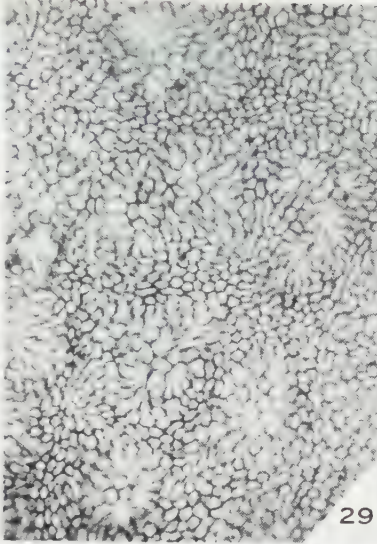
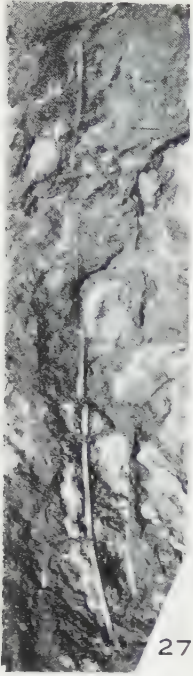
COOKSON AND DUIGAN.—FOSSIL BANKSIEAE FROM YALLOURN, VICTORIA

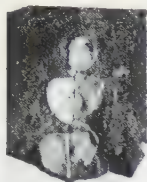


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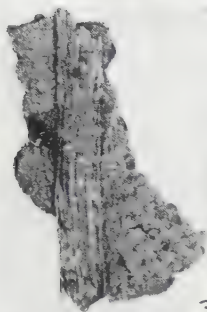


COOKSON AND DUIGAN.—FOSSIL BANKSIEAE FROM YALLOURN, VICTORIA





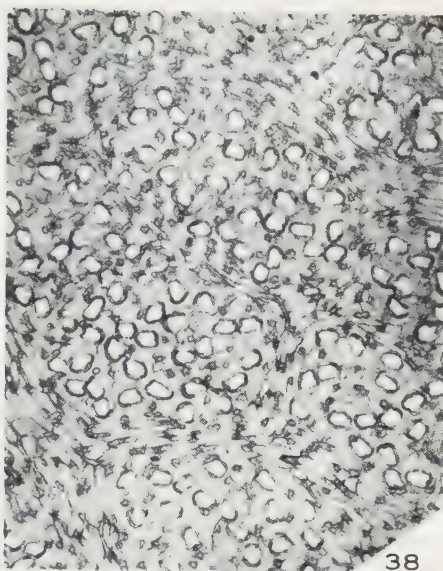
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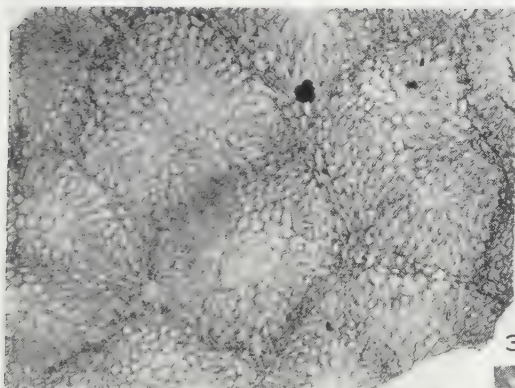
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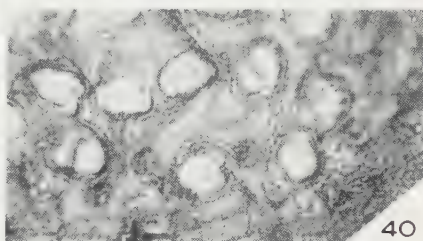
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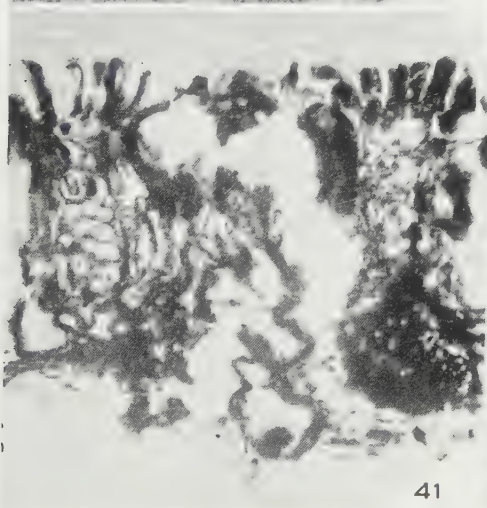
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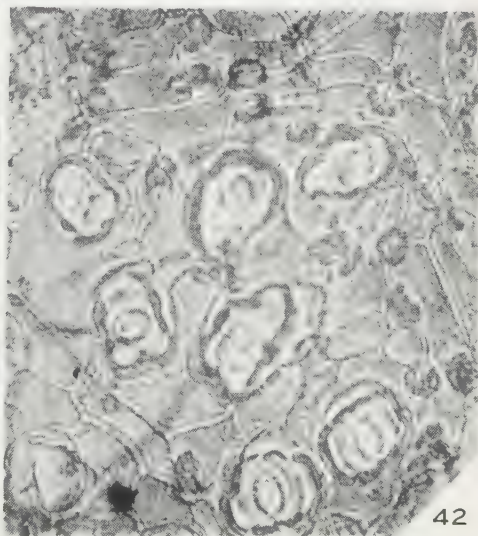
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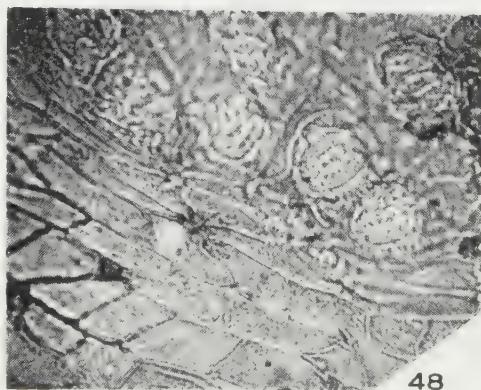
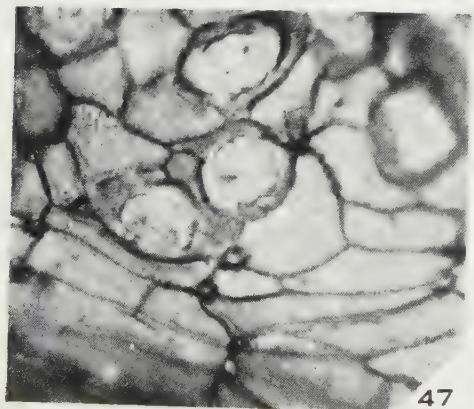
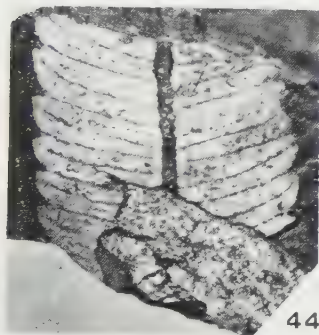
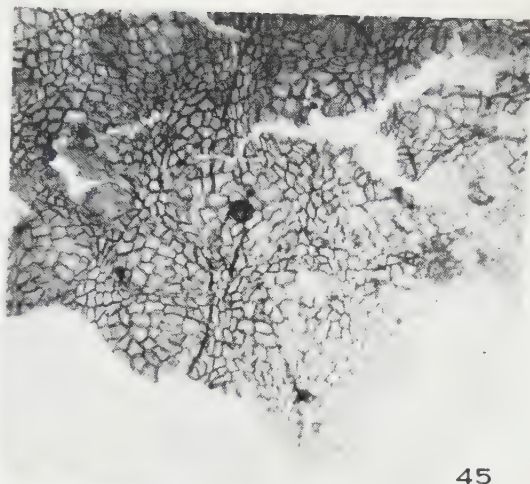
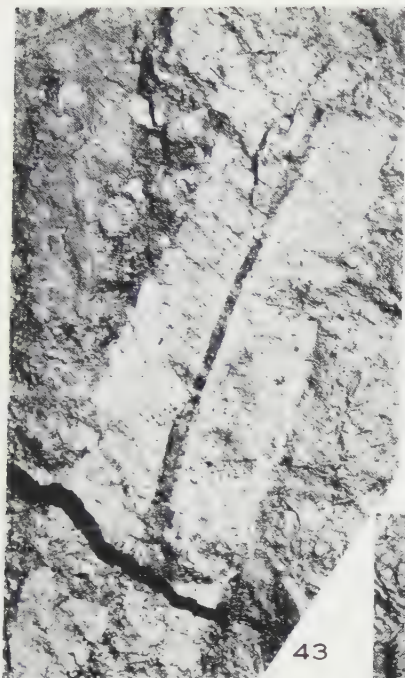
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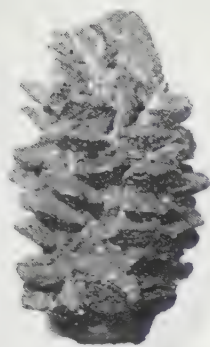


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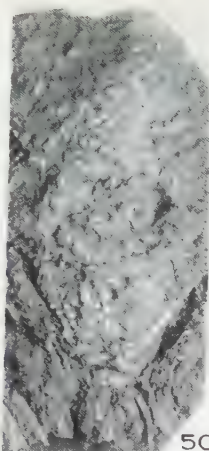


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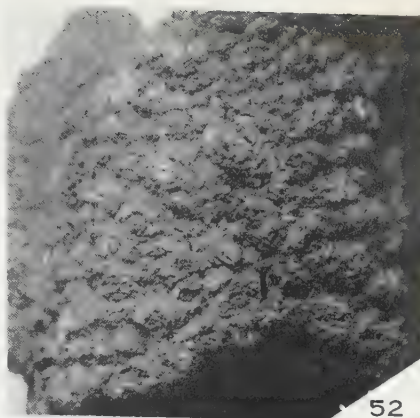




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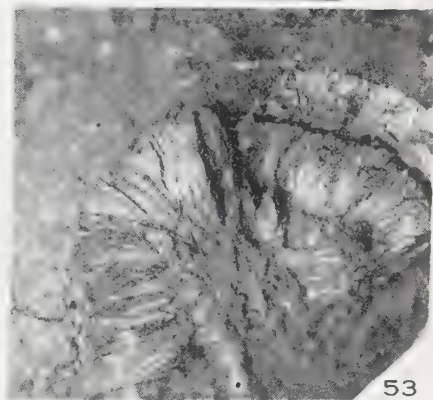
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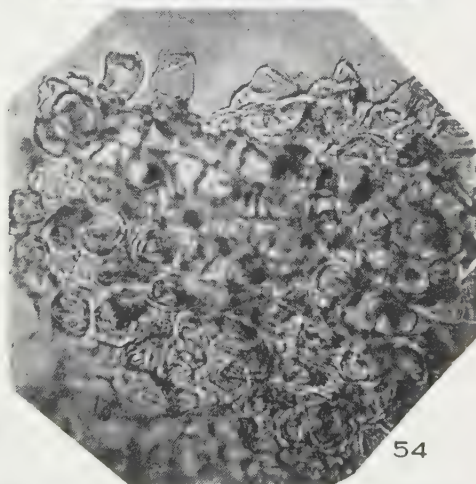
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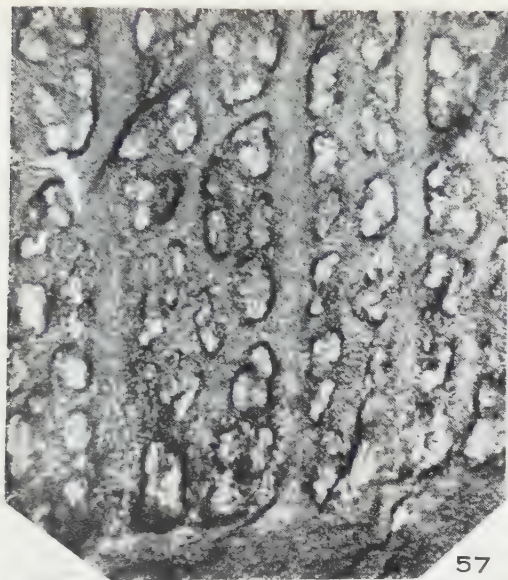
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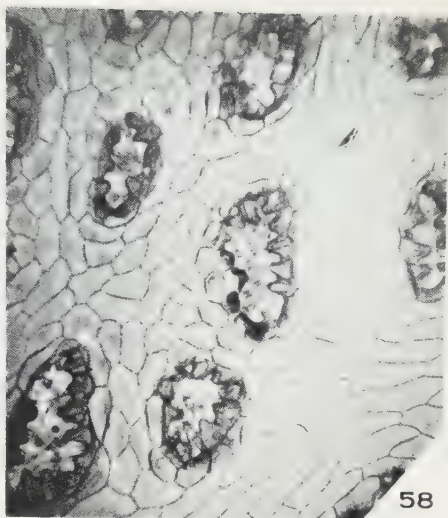
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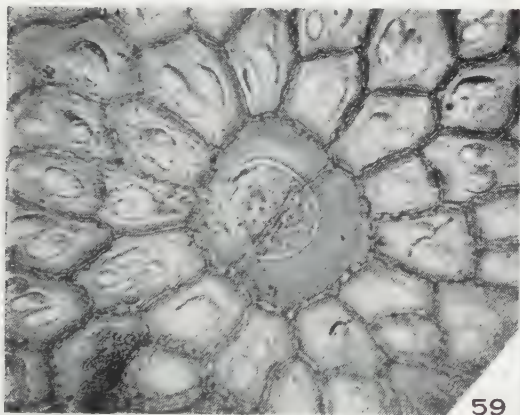
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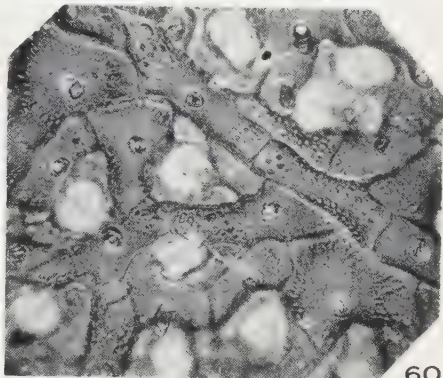
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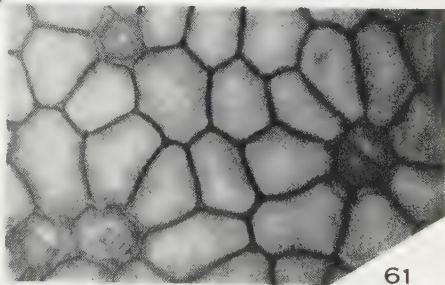
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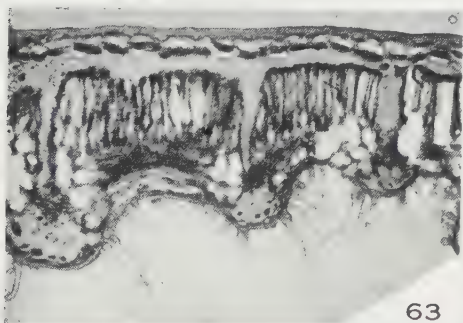
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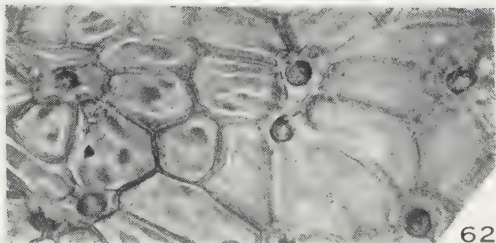
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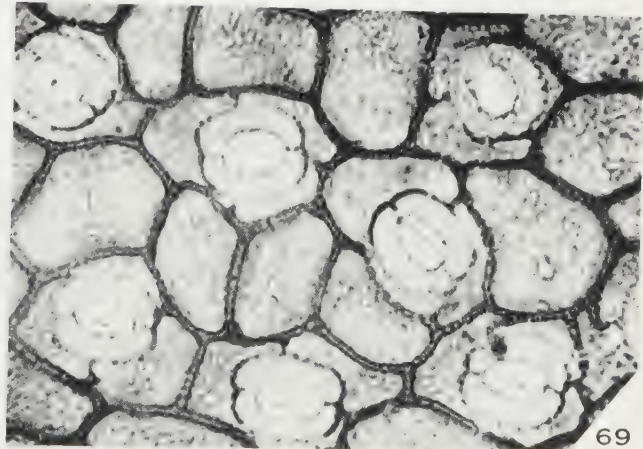
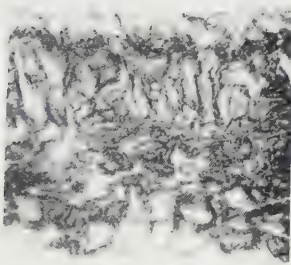
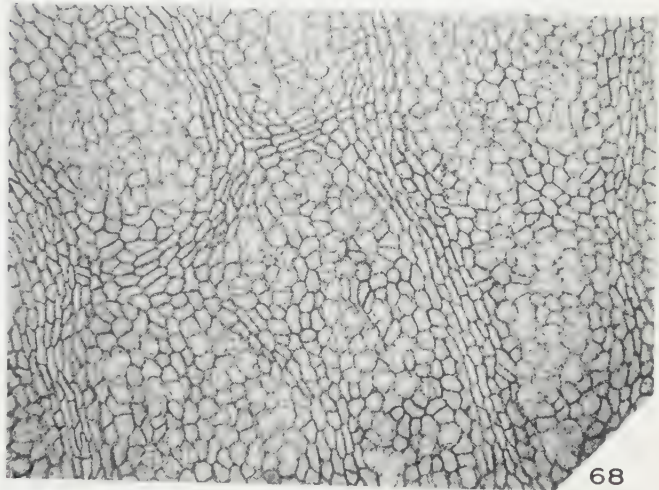
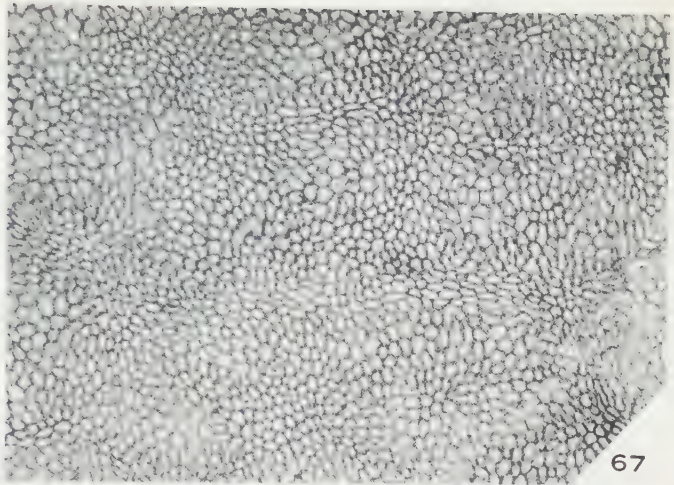
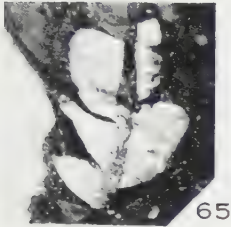
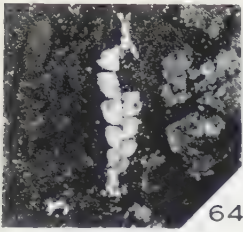
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- Fig. 59.—Cuticle of upper epidermis of *Dryandra floribunda* showing radial arrangement of epidermal cells around two cells supporting a large hair-base, their strongly pitted walls, and large cuticular papillae. x 325.
- Fig. 60.—Cuticle of lower epidermis of *Dryandra kippistiana* showing numerous small cuticular papillae both on cells with and without hair-bases. x 230.
- Fig. 61.—Cuticle of upper epidermis of *Banksia benthamiana* showing un-uniform, unpitted parenchyma cells and small hair-bases supported by one or two equally small cells. x 450.
- Fig. 62.—Cuticle of upper epidermis of *Banksia baueri* showing the cells of two sizes which comprise the epidermal parenchyma. The small cells support small hair-bases. x 300.
- Fig. 63.—Transverse section of *Banksia robur* showing two-layered hypoderm, vertical plates of sclerenchymatous fibres, stomatal pits, and hairs. x 60.

PLATE 9

Phyllites yallournensis n.sp.

- Fig. 64.—Basal portion of a leaf from above. Natural size. (N.M.V. No. 14777.)
- Fig. 65.—Under surface of another fragment. (N.M.V. No. 14778.)
- Fig. 66.—A leaf fragment from above. x 3. (N.M.V. No. 14779.)
- Fig. 67.—Cuticle of upper epidermis. x 100.
- Fig. 68.—Cuticle of lower epidermis. x 100.
- Fig. 69.—Cuticle of lower epidermis. x 420.
- Fig. 70.—Cross section of leaf. x 250.

FOSSIL POLLEN GRAINS OF PROTEACEOUS TYPE FROM TERTIARY DEPOSITS IN AUSTRALIA

By ISABEL C. COOKSON*

[Manuscript received March 22, 1950]

Summary

Seventeen sporomorphs of possible proteaceous affinities have been described from Tertiary (?Oligocene – Miocene) deposits of south-eastern Australia. The majority of these appear to have been pollen grains of extinct species.

The sporotypes *Banksieacidites* and *Beaupreaidites*, each represented by two sporomorphs, are morphologically identical with pollen grains of the tribe Banksieae and two species of *Beauprea* (*B. elegans* and *B. spathulaefolia*) respectively.

The resemblance between the sporomorph *Proteacidites annularis* and pollen of *Xylomelum occidentale*, a species now confined to Western Australia, is discussed. Attention is drawn to the likeness of the sporomorph *Proteacidites symphyonemoides* to pollen of *Symphyonema*; *Proteacidites truncatus* to pollen of *Isopogon* and *Proteacidites adenanthoides* to pollen of *Adenanthos barbigera*.

A morphologically distinct pollen type from brown coal at Moorlands in South Australia is described under the sporomorph *Triorites magnificus*.

I. INTRODUCTION

A variety of pollen grains which are morphologically conformable with those of living members of the family Proteaceae occur in a number of Australian Tertiary deposits (see Pike 1948). This, at first sight, may appear surprising since pollination in this family normally takes place through the agency of insects and birds (Bentham 1873; Müller 1883; Brough 1933; Carey 1941). A small amount of scattering by wind apparently does take place since Phillips (1941) in a paper dealing with atmospheric pollen caught in and about Sydney, New South Wales, reports that "a few pollen grains of Proteaceae have been caught from time to time" and notes that "the highest percentage was 1.2 from Parramatta." During the present investigation this question was attacked from a different angle (cf. Carroll 1943) with similar results. A pollen analysis of lichen thalli taken from an area rich in Proteaceae near Perth, Western Australia, was carried out. The material, kindly supplied by Dr. B. J. Grieve, was subjected to acetolysis and the spores which had collected on the surface of the thalli were concentrated and mounted in the usual manner. Examination of the concentrate revealed the presence of numerous fern spores, pollen grains of Myrtaceae, Compositae, some monosulcate types, and a low percentage of proteaceous pollen grains. These included a single grain of *Isopogon* sp., and a few grains of a species of *Adenanthos*.

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Such results, as far as they go, suggest that pollen grains of the Proteaceae are unlikely to be major constituents of spore-bearing deposits. They do not, however, preclude the possibility of their presence in smaller numbers. Furthermore, clusters of pollen grains can usually be found on the central stigmatic processes of fully opened and fading flowers, so that if such flowers or floral parts were to fall into developing sediments they would clearly provide an additional source of pollen grains for later fossilization.

The present paper deals with some of the more conspicuous proteaceoid types occurring in Australian Tertiary deposits. Little or no doubt exists as to the family or even generic relationships of some of these, but the affinity of others is less certain.

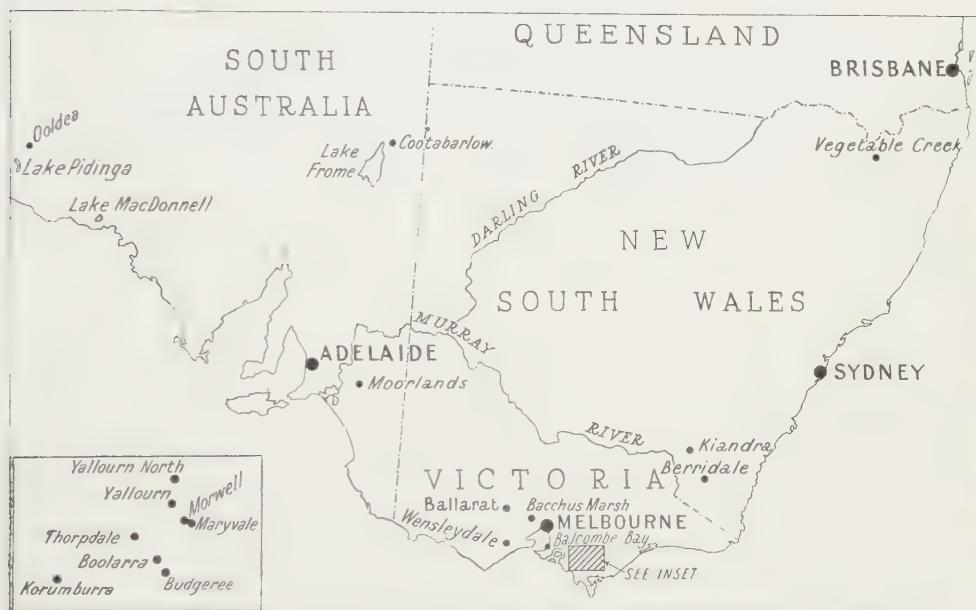


Fig. 1.—Map of south-eastern Australia showing location of deposits in which proteaceous-like pollen has been found. (Prepared by the Geological Survey of Victoria.)

The localities of most of the deposits which will be referred to have already been mentioned in a previous paper (Cookson 1946). To these six new localities (all of which are shown in the accompanying map) have now to be added, namely:

Yallourn North—Open cut in brown coal, immediately north of the Latrobe River at Yallourn, Victoria.

Wensleydale—About 10 miles north-west from Anglesea, Victoria.

Bacchus Marsh—Lucifer Mine, half a mile south-east of Bacchus Marsh railway station, Victoria.

Lake MacDonnell—Close to the sea on the west coast of Eyre Peninsula, about 8 miles south of Penong, the terminal station of the 3 ft. 6 in. gauge railway line and 63 miles distant from Thevenard, South Australia.

Pidinga—About 35 miles south-east of Ooldea, a siding on the east-west railway line 427 miles from Port Augusta, South Australia.

Cootabarlow—At Lake Cootabarlow, east of and near Lake Frome, South Australia.

The system of nomenclature followed is that proposed by Erdtman in 1947 for fossil pollen grains and spores and adopted by Cookson (1947) and Ross (1949). The terms used in the pollen morphological descriptions are after Erdtman (1948).

Measurements and photographs of the fossil pollen grains haven been taken from material which, with one exception, has been prepared by the use of the acetolysis method. A 12½ per cent. solution of sodium hypochlorite has been found useful for the preliminary examination of sediments and for the rapid checking of results. If necessary, it may be preceded by treatment with hydrofluoric acid.

II. DESCRIPTIONS OF POLLEN GRAINS STUDIED

BEAUPREIDITES n. spt.

Plate 1, Figs. 2, 4, 6, 7

Although occurring in small numbers only, this sporotype has been observed in preparations from several widely separated Australian Tertiary deposits. It is characterized by its medium size, straight sides, colpoid apertures, tapering exine, and finely reticulate sexine. In all these features it shows a remarkable resemblance to the pollen of two species of the New Caledonian genus *Beauprea*. In fact, had the colpoid type of aperture not already been observed in the Proteaceae by its occurrence in *Beauprea*, the proteaceous affinity of this sporotype might not have been recognized. As the result of a comparison of the fossil and living pollen grains it now appears probable that species of *Beauprea* were components of the Tertiary flora of southern Australia.

BEAUPREIDITES ELEGANSIFORMIS n. spm.

Plate 1, Figs. 2-4

Grains.—Triangular with straight sides and rounded angles; equatorial diameter 36-52 μ , average 45 μ ; apertures colpoid, sometimes with irregular slits which extend beyond the apertural regions.

Exine.—About 2.3 μ , tapering towards the apertures; sexine and nexine of about equal thickness.

Sexine.—Baculate, reticulate, mesh of reticulum small, becoming considerably reduced towards the apertural areas, the latter lighter in colour in both

stained and unstained preparations as in pollen of the living species *Beauprea elegans* Brongn. et Gris (Plate 1, Fig. 1) and *Beauprea spathulaefolia* Brongn. et Gris; muri psilate.

Affinity.—Closely similar in all respects to pollen of *Beauprea elegans*.

Distribution.—Widely distributed in south-eastern Australia.

Brown coal: Yallourn open cut, 32 and 62 ft. from top of coal; Moorlands; Lake MacDonnell bore 213 ft. 6 in.-217 ft. (Preparation by K. Pike.)

Ligneous clay: Lucifer Mine, Bacchus Marsh; Hoddles Creek, Victoria.

Mudstone: Vegetable Creek.

BEAUPREAIDITES VERRUCOSUS n. spm.*

Plate 1, Figs. 6, 7

This sporomorph conforms to the description given above for *B. elegansiformis* but differs from it in the presence of small, irregularly disposed thickenings on the surface of the sexinous reticulum. Similar localized thickenings of the muri occur in the majority of pollen grains of *Beauprea spathulaefolia* (Plate 1, Fig. 5) so that the establishment of a second sporomorph, on this feature alone, appears to be justified.

Affinity.—Closest morphological agreement is with pollen of *Beauprea spathulaefolia*.

Distribution.—*Ligneous clay*: Lucifer Mine, Bacchus Marsh; Cootabarlow Bore.

BANKSIEAEIDITES n. spt.

Pollen grains of this sporotype are sub-isopolar, bilateral, biaperturate; the ectonexine is thickened around the apertures. They closely resemble pollen grains of living species of *Banksia* and *Dryandra* which comprise the tribe Banksieae, and there is little doubt that they belonged to Tertiary members of this tribe. Since, however, the pollens of *Banksia* and *Dryandra* are indistinguishable from one another, the fossil grains cannot be attributed to either genus.

BANKSIEAEIDITES MINIMUS n. spm.

Plate 1, Figs. 18, 19

Grain.—Plano-convex, length 24-35 μ , breadth 16-24 μ (occasionally as broad as long), width 19-21 μ ; apertures 8-11 μ .

Exine.—Thin, 1.5 μ , apertural "collars" 2.5-3 μ deep.

Sexine.—Sculpture faint, probably a thick-walled reticulum.

Distribution.—*Brown coal*: Open cut, Yallourn North; open cut, Yallourn, at 62 and 92 ft. from top of coal.

* The southern distribution of *B. verrucosus* has recently been extended by its discovery in lignite from Ouse, Tasmania, the latitude of which is 42° 48'S.

BANKSIEAEIDITES ELONGATUS n. spm.

Plate 1, Fig. 10

Grain.—Plano-convex; length 40-56 μ , breadth 20-27 μ ; apertures 8-11 μ .

Exine.—About 2 μ thick, "collars" 5 μ deep.

Sexine.—Sculpture more clearly defined than in *B. minima*, probably a thick-walled reticulum.

Distribution.—*Brown coal*: Open cut, Yallourn North; Yallourn open cut at 62-92 ft. from top of coal.

Grains of *Banksieaeidites* have been observed in preparations of Moorlands coal and other deposits but their numbers are as yet too small for them to be reliably identified with either of the sporomorphs from Yallourn.

PROTEACIDITES TUBERCULATUS n. spm.

Plate 1, Figs. 12-14

Grain.—Large, triangular, with convex sides and concave apertures, the average equatorial diameter being 78 μ , the range from 60 to 96 μ .

Exine.—About 5.5 μ , tapering towards the apertures and showing clearly defined nexinous and sexinous layers; the ectonexine represents at least 80 per cent. of the total thickness of the exine and the endonexine is sharply defined.

Sexine.—Very thin, baculate, covered with approximately spherical tubercles, 3-4 μ high, which are arranged either irregularly or in a reticuloid pattern. Both the proximity and size of tubercles vary considerably in grains from different localities. The number of specimens observed, however, is, as yet, too small to permit a less generalized statement.

Affinity.—In spite of the distinctive form of *P. tuberculatus*, it cannot be matched with pollen of any living proteaceous species. It shows a superficial resemblance to pollen of *Beauprea balansae* Brongn. et Gris from New Caledonia (Plate 1, Fig. 11) but the smaller size, colpoid apertures, and reticulate sexine of the latter are important contrasting features. The sculpture of *P. tuberculatus* recalls, in slight measure, that of *Hakea pycnoneura* and *Hakea petiolaris* but the shapes of the grains and apertures do not agree with those of pollens of *Hakea*.

Distribution.—*Brown coal*: Yallourn open cut at 32 and 92 ft. below top of coal; Moorlands.

Ligneous clay: Lucifer Mine, Bacchus Marsh.

PROTEACIDITES ANNULARIS n. spm.

Fig. 2 and Plate 1, Fig. 15

Grains.—Triangular with prominent apertural areas; sides straight to concave, apertures 5.8 μ ; average equatorial diameter 32 μ with a range of 27 to 37 μ .

Exine.— $2.76\ \mu$ thick, clearly differentiated into three layers. At the base of each apertural area an abrupt annular thinning of the ectonexine is followed by a pronounced thickening of the same layer which then gradually tapers towards the opening. This thickened area forms a "collar" around the aperture, the depth of which is about $5\text{--}6\ \mu$; frequently the thinner portion beyond the "collar" is not preserved.

Sexine.—About half as thick as the nexine, sculpture in the form of a very fine reticulum.

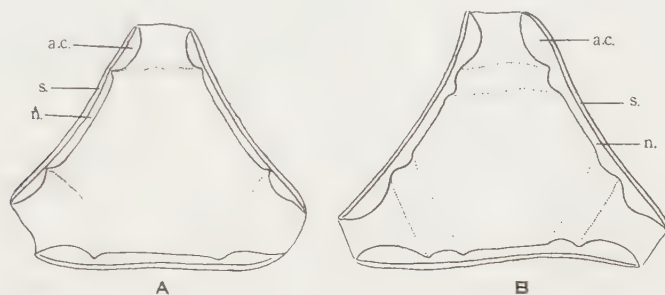


Fig. 2.—Camera lucida drawings of pollen grains of (A) *Proteacidites annularis* and (B) *Xylomelum occidentale*.

s., sexine; n., nexine; a.c., apertural "collar." $\times 835$.

Affinity.—*P. annularis* probably recurs in the preparations more often than any other proteaceous pollen. It is readily identified by its apertural areas and "collars." Amongst recent species of Proteaceae similar features are most strongly marked in pollen of *Lambertia* Sm., *Xylomelum angustifolium* Kipp., *Xylomelum occidentale* R. Br., *Banksia* Linn., and *Dryandra* R. Br. The fossil grains being radiosymmetrical and triaperturate, comparison can be restricted to *Lambertia* and the two species of *Xylomelum* mentioned. Of the *Lambertias*, pollen of *Lambertia multiflorum* is most like the sporomorph *P. annularis*, but the "collars" of the latter are more strongly defined and the grains do not appear to be sub-isopolar.

As regards the two species of *Xylomelum*, so close is the resemblance between the fossil grains and pollen of *X. occidentale* (Fig. 2 and Plate 1, Fig. 16) that a relationship between the two parent plants seems probable.

Distribution.—The most widely distributed and most numerous type.

Brown coal: Yallourn open cut, 32, 62, and 92 ft. from top of coal; Hoddles Creek, Victoria; Lucifer Mine, Bacchus Marsh; Moorlands; Cootabarlow Bore at 537-538 ft.

Ligneous clay: Lal Lal, near Ballarat; Wensleydale (G.S.V. 50083).

Mudstones: Balcombe Bay; Vegetable Creek.

PROTEACIDITES SYMPHYONEMOIDES n. spm.

Plate 2, Fig. 17

Grain.—Triangular with straight or slightly convex sides narrowing somewhat towards the apertures; equatorial diameter averaging 32 μ and ranging from 24 to 37 μ ; apertures about 3 μ .

Exine.—Thin, approximately 1.5 μ ; not tapering towards the apertures.

Sexine.—Thicker than the nexine, baculate, reticulate; the muri have wavy outlines and the meshes are of irregular sizes and shapes.

Affinity.—This type approaches most nearly the pollen of the recent genus *Symphyonema* R. Br. The size of the grains and degree of coarseness of the sexinous reticulum appear to be intermediate between those of *S. paludosum* R. Br. and *S. montanum* R. Br.

Distribution.—*Ligneous clay*: Yallourn open cut, fruit zone collected by Dr. Austin Edwards.

Ligneous shale: Kiandra, C.S. 89.

Mudstone: Balcombe Bay.

PROTEACIDITES TRUNCATUS n. spm.

Plate 2, Fig. 19

Grain.—Triangular with straight sides and transversely extended apertures; average equatorial diameter 46 μ .

Exine.—About 3 μ , tapering towards the apertures.

Sexine.—Baculate, reticulate, almost as thick as the nexine; mesh moderately large, becoming reduced towards the apertures.

Affinity.—*P. truncatus* is a rare type with characters which resemble those of *Isopogon* R. Br. The most striking feature of agreement, apart from the sexinous reticulum, is the equatorial extension of the apertures which give a truncate appearance to the angles of the grains. Of the recent species examined, the one with which the closest comparisons are possible is *I. anemonifolius* Knight (Plate 2, Fig. 18), an eastern Australian representative.

Distribution.—*Brown coal*: Yallourn open cut, at 32 and 62 ft. from top of coal.

PROTEACIDITES ADENANTHOIDES n. spm.

Plate 2, Fig. 21

Grain.—Triangular, slightly sub-isopolar, sides more or less concave according to the position in which the grain is viewed; average equatorial diameter 43 μ , range 32-48 μ ; diameter of the apertures about 3 μ .

Exine.—2.8 μ thick, nexine not thinning towards the apertures.

Sexine.—About half as thick as the nexine, widest between the apertures, reticulate. Reticulum baculate, practically identical in both hemispheres, mesh small, becoming finer towards the apertures, muri rather thick.

Affinity.—This sporomorph closely resembles pollen of the living genus *Adenanthos*, particularly that of *Adenanthos barbiger* (Plate 2, Fig. 20), both in form and type of sexinous reticulum. When closely compared with the latter, however, *B. adenanthoides* is seen to be less sub-isopolar with the width of the mesh the same over both poles and to have a thicker exine.

Distribution.—*Ligneous clays*: Wensleydale (G.S.V. No. 50083); Cootabarlow bore 493-515 ft., 515-537 ft.

Mudstone: Vegetable Creek

PROTEACIDITES CRASSUS n. spm.

Plate 2, Fig. 22

Grain.—Three or sometimes two-aperturate, sub-isopolar, plano-convex, average equatorial diameter 58 μ , polar axis about 27 μ , sides concave.

Exine.—2.3 μ , nexine not thinning towards the apertures.

Sexine.—Reticulate, slightly more than half as thick as nexine, widest between the apertures. Mesh of reticulum moderately large, finer in the convex hemisphere and towards the apertures, muri thick, baculae clearly defined.

Affinity.—The most conspicuous difference, apart from size, between this and the preceding sporomorph is the coarser reticulation and the stronger sub-isopolarity reflected in the greater width of mesh in the concave hemisphere. In this respect *P. crassa* is more “*adenanthoid*” than *P. adenanthoides*. It is regarded as pollen of an extinct proteaceous plant.

Distribution.—*Brown coal*: Moorlands.

Ligneous clay: Cootabarlow bore, 493-515 ft., 515-537 ft.

PROTEACIDITES GRANDIS n. spm.

Plate 2, Fig. 23

Grain.—Slightly sub-isopolar, more or less triangular in polar view, usually with deeply concave sides; large, the average equatorial diameter being 69 μ and the polar axis approximately 27 μ .

Exine.—3.7 μ thick, gradually thinning towards the apertures.

Sexine.—Baculate, about as thick as the nexine, reticulate; the reticulum similar in both hemispheres with wide lumina and narrow muri, becoming minute towards the apertures.

Affinity.—The affinities of this sporomorph are obscure. It is unlike pollen of living proteaceous species. As regards the fossil sporomorphs a general likeness to *P. crassa* may be mentioned but in size, the degree of sub-isopolarity, and coarseness of the sexinous reticulum, the two are distinct.

Distribution.—*Ligneous clay*: Wensleydale (G.S.V. 50083).

PROTEACIDITES RETICULATUS n. spm.

Plate 1, Fig. 24

Grain.—Triangular, sub-isopolar, plano-convex, sides usually deeply concave; size medium, average $27 \times 41 \mu$ oblate.

Exine.—Thin, 2.3μ , tapering towards the apertures.

Sexine.—In the form of a delicate, wide-meshed reticulum which becomes very fine near the apertures; the lumina are wider and the muri thicker in the concave hemisphere; the muri are composed of pilate elements which sometimes appear to form a minute mural reticulum.

Affinity.—The affinity of this sporomorph is obscure.

Distribution.—*Brown coal*: Moorlands.

PROTEACIDITES INCURVATUS n. spm.

Plate 2, Figs. 25, 26

Grain.—Large, triangular, with more or less deeply concave sides; range in equatorial diameter from 44 to 80μ ; apertures from 14 to 20μ , slightly concave.

Exine.—Thick, 3.5 - 5μ , tapering and curving in towards the apertures.

Sexine.—About as wide as the nexine, baculate, somewhat reticuloid at high focus, pilate groups becoming smaller towards the apertures.

Affinity.—Although the general morphology of this sporomorph points distinctly towards the Proteaceae, it is not definitely comparable with pollens of any living species. The clearly marked tapering and incurving of the exine towards the apertures distinguishes *P. incurvatus* from the simpler pollen grains of *Hakea* and *Grevillea*. The examples from Wensleydale and Pidinga (Plate 2, Fig. 26) are somewhat smaller and the pilate groups of the sexine coarser than in the grains from Moorlands.

Distribution.—*Brown coal*: Moorlands, South Australia; Pidinga, South Australia, bore at 35 ft. 6 in. to 41 ft. 6 in.

Ligneous clay: Wensleydale (G.S.V. No. 50083).

PROTEACIDITES RECTOMARGINIS n. spm.

Plate 2, Fig. 27

Grain.—Large, triangular, sides straight; equatorial diameter 64μ , range 50 - 80μ ; apertures slightly concave, about 14μ .

Exine.— 3.5μ , of even width throughout.

Sexine.—About the same width as the nexine; baculate, pilate groups approximately the same size throughout. Sometimes the smooth nexinous surface is exposed by the partial or complete destruction of the sexine.

Affinity.—In general morphology this sporomorph resembles pollen grains of *Petrophila media* and *Petrophila ericifolia*. In both these species the grains are large, triangular, with straight sides, and slightly concave apertures. There

appears to be a divergence, however, when the sexinous patterns are compared. The question of the affinity of *P. rectomarginis* must therefore remain open.

Distribution.—*Ligneous clay*: Lucifer Mine, Bacchus Marsh, Victoria.

PROTEACIDITES CALLOSUS n. spm.

Plate 3, Fig. 28

Grain.—Triangular with straight sides; average equatorial diameter 41 μ , range 35-48 μ .

Exine.—2.5 μ thick, ectonexine thickened at the apertures.

Sexine.—Slightly thicker than the nexine, baculate, reticulate, mesh fine, irregular, muri thick.

Distribution.—*Brown coal*: Moorlands.

PROTEACIDITES PARVUS n. spm.

Plate 3, Fig. 29

Grain.—Triangular, sides straight; equatorial diameter about 35 μ ; apertures slightly concave.

Exine.—1.8 μ , of even thickness throughout.

Sexine.—Slightly thicker than nexine; finely baculate.

Distribution.—*Mudstone*: Vegetable Creek.

PROTEACIDITES OBSCURUS n. spm.

Plate 3, Figs. 30, 31

Grain.—Triangular, sides straight to concave; average equatorial diameter 38 μ , range 35-43 μ .

Exine.—4 μ thick, ectonexine prominent, slightly thickened towards the apertures where it appears lancet-shaped in optical section (Plate 3, Fig. 30).

Sexine.—Delicate, thinner than nexine; widely baculate and somewhat reticuloid except near the apertures where it is thicker and more homogeneous. Frequently the sexine covering the general body of the grain is only partially (Plate 3, Fig. 31) or not at all preserved.

Affinity.—Obscure. It may represent the pollen of an extinct proteaceous plant but is unlike that of any of the living species.

Distribution.—*Brown coal*: Moorlands; Yallourn, 32 ft. from top of coal.

Mudstone: Balcombe Bay.

TRIORITES MAGNIFICUS n. spm.

Plate 3, Figs. 32-35

This pollen grain in its triangular, triaperturate form superficially resembles pollen of the Proteaceae, but when its general morphology and sclerine stratification are examined unusual features become evident. For the present it

seems advisable to regard it as having belonged to an extinct plant of uncertain affinities and to refer it to the sporotype *Triorites*. It is described here partly because of its interest from a pollen morphological point of view and partly for its possible usefulness as a zone fossil

Grain.—Large, $55 \times 71 \mu$, oblate, the equatorial diameter ranging from 53 to 88μ ; triangular, with straight to slightly concave sides.

Exine.—Thick, $9-10.5 \mu$, composed of four clearly defined layers. Of these the two inner are nexinous (ecto and endonexine) with an approximate width of 2.5μ and the two outer are sculptinous (sexine and ?perine). The inner layer of the sculptine is broad, about 4.5μ , and has a pitted or somewhat spongy consistency. The outer layer of the sculptine, which is about 2.5μ thick, is highly refractive and does not take the stain (basic fuchsin) like the rest of the exine. It is baculate and in surface view appears reticuloid (Plate 3, Fig. 33). In some specimens it is partially or completely destroyed, thus exposing the "spongy" layer (Plate 3, Fig. 35).

Another unusual feature of this sporomorph is the position of the true apertures. These appear to be situated at the bases of cavities formed by forward annular extensions of the exine. Moreover, the sculptinous layers turn in to line these extra-apertural cavities, thereby completely enveloping the distal extensions of the ectonexine.

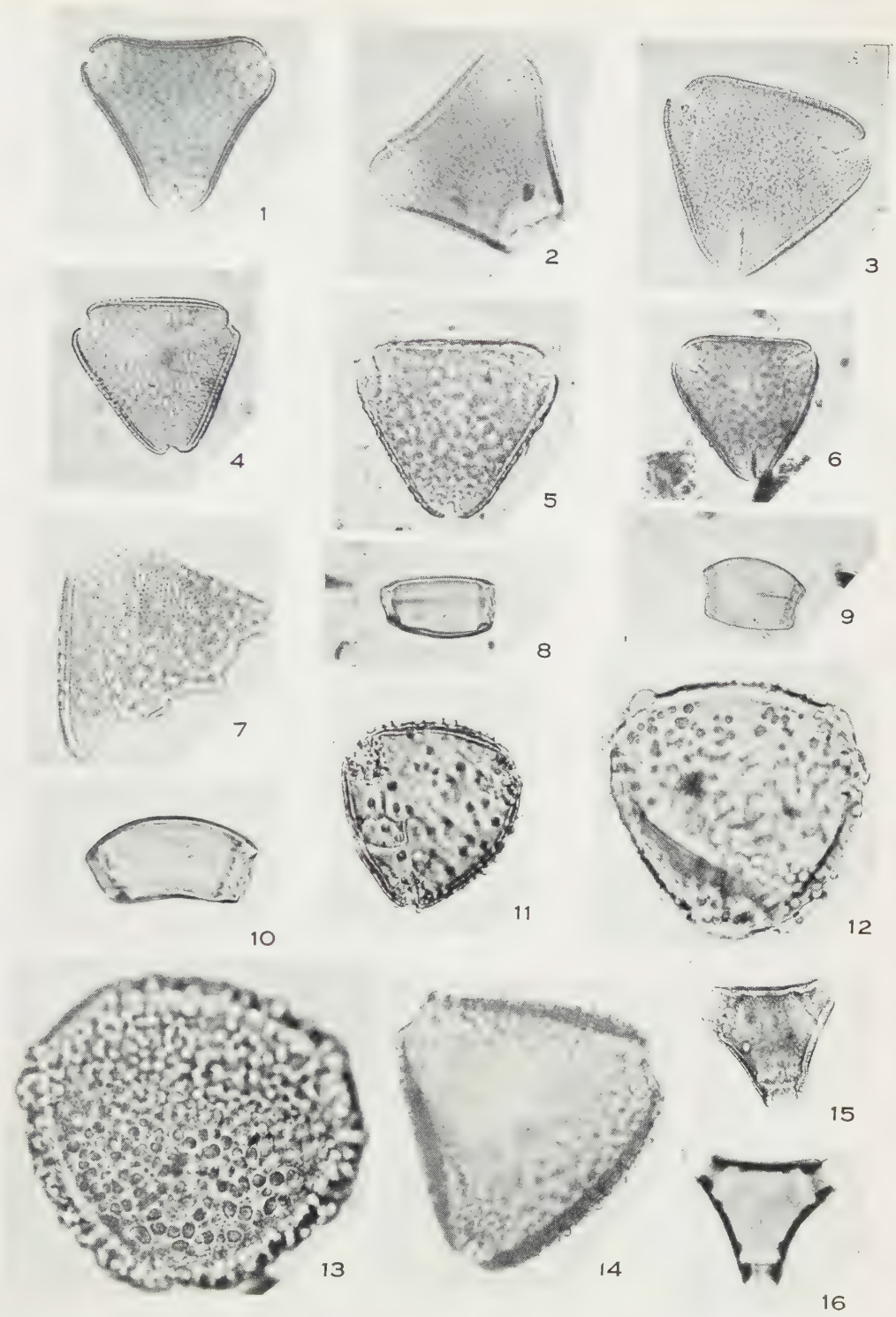
Distribution.—*Brown coal*: Moorlands.

III. ACKNOWLEDGMENTS

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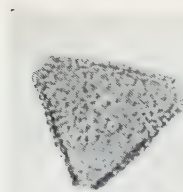
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COOKSON.—FOSSIL POLLEN GRAINS OF PROTEACEOUS TYPE FROM TERTIARY DEPOSITS
IN AUSTRALIA

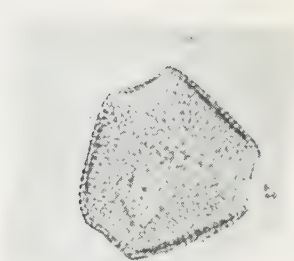




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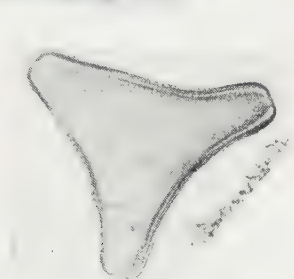
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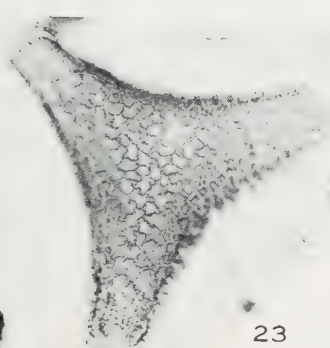
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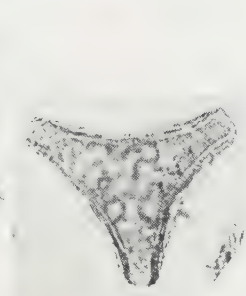
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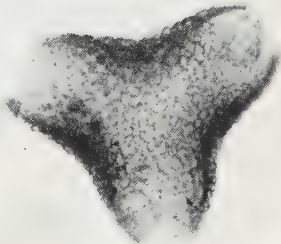
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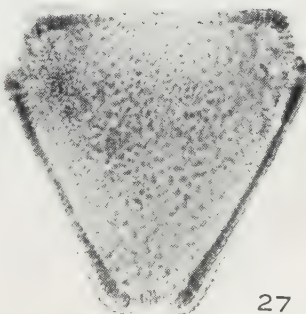
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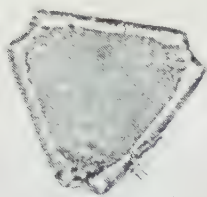


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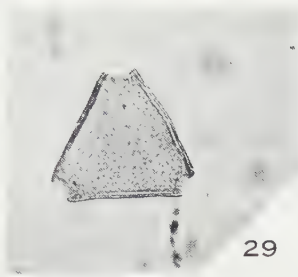


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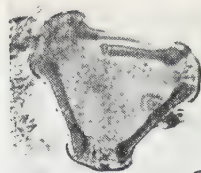




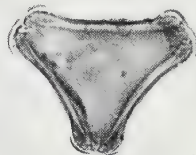
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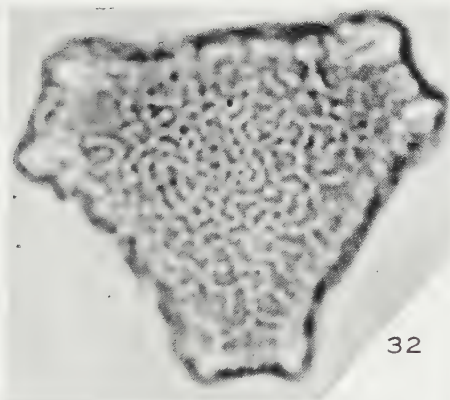
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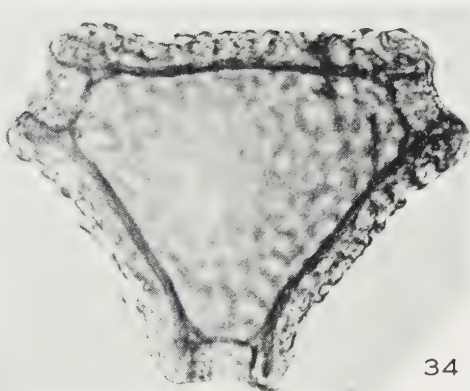
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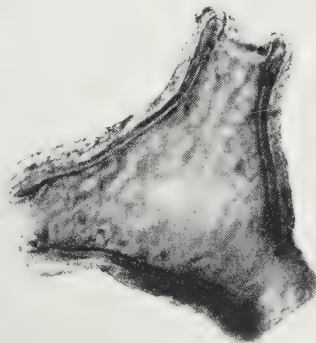
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COOKSON.—FOSSIL POLLEN GRAINS OF PROTEACEOUS TYPE FROM TERTIARY DEPOSITS
IN AUSTRALIA

EXPLANATION OF PLATES 1-3

All the figures are from untouched negatives; the pollen grains are magnified 500 diameters.

PLATE 1

- Fig. 1.—Pollen grain of *Beauprea elegans*, New Caledonia.
 Fig. 2.—*Beaupreadites elegansiformis* n. spm., Bacchus Marsh, Victoria.
 Fig. 3.—*Beaupreadites elegansiformis* n. spm., Yallourn, Victoria, 32 ft. below top of coal.
 Fig. 4.—*Beaupreadites elegansiformis* n. spm., Moorlands, South Australia.
 Fig. 5.—Pollen grain of *Beauprea spathulaefolia*, New Caledonia.
 Fig. 6.—*Beaupreadites verrucosus* n. spm., Wensleydale, Victoria.
 Fig. 7.—*Beaupreadites verrucosus* n. spm. Fragment of a large grain to show roughened surface of sexinous reticulum.
 Figs. 8 and 9.—*Banksiaeaidites minimus* n. spm., Yallourn North, Victoria.
 Fig. 10.—*Banksiaeaidites elongatus* n. spm., Yallourn North, Victoria.
 Fig. 11.—Pollen grain of *Beauprea balansae*, New Caledonia.
 Fig. 12.—*Proteacidites tuberculatus* n. spm. Grain in optical section showing (on the left-hand side) thick ectonexine, thin sexine and ?perinous tubercles. Yallourn, Victoria.
 Fig. 13.—*Proteacidites tuberculatus* n. spm. A large example focused to show tubercles and baculae of sexine. Yallourn, Victoria.
 Fig. 14.—*Proteacidites tuberculatus* n. spm., Lucifer Mine, Bacchus Marsh.
 Fig. 15.—*Proteacidites annularis* n. spm., Moorlands, South Australia.
 Fig. 16.—Pollen grain of *Xylomelum occidentale*, Western Australia.

PLATE 2

- Fig. 17.—*Proteacidites symphyonemoides* n. spm., Yallourn, Victoria.
 Fig. 18.—Pollen grain of *Isopogon anemonifolius*, New South Wales.
 Fig. 19.—*Proteacidites truncatus* n. spm., Yallourn, Victoria.
 Fig. 20.—Pollen grain of *Adenanthos barbigera*, Western Australia.
 Fig. 21.—*Proteacidites adenanthoides* n. spm., Wensleydale, Victoria.
 Fig. 22.—*Proteacidites crassus* n. spm., Moorlands, South Australia.
 Fig. 23.—*Proteacidites grandis* n. spm., Wensleydale, Victoria.
 Fig. 24.—*Proteacidites reticulatus* n. spm., Moorlands, South Australia.
 Fig. 25.—*Proteacidites incurvatus* n. spm., Moorlands, South Australia.
 Fig. 26.—*Proteacidites incurvatus* n. spm., Pidinga, South Australia.
 Fig. 27.—*Proteacidites rectomarginis* n. spm., Bacchus Marsh, Victoria.

PLATE 3

- Fig. 28.—*Proteacidites callosus* n. spm., Moorlands, South Australia.
 Fig. 29.—*Proteacidites parvus* n. spm., Vegetable Creek, New South Wales.
 Fig. 30.—*Proteacidites obscurus* n. spm., Moorlands, South Australia.
 Fig. 31.—*Proteacidites obscurus* n. spm. A grain with sexine preserved only around the apertures. Moorlands, South Australia.
 Figs. 32-34.—*Triorites magnificus* n. spm. A grain focused at three different levels. Moorlands, South Australia.
 Fig. 35.—*Triorites magnificus* n. spm. A grain from which the outer layer of sculptine has almost entirely disappeared. The complete thickness of the exine can be seen on the left-hand side near the upper aperture.

THE INFLUENCE OF RAINFALL ON THE YIELD OF WHEAT IN SOUTH AUSTRALIA

By E. A. CORNISH*

[*Manuscript received December 20, 1949*]

Summary

The relationship between the yield of wheat and seasonal rainfall in South Australia has been determined. The period chosen for examination was 1896-1941 and the analysis extends to practically the entire wheat belt of the State, all major soil groups and variants of climatic conditions within the area being represented. Yield was assessed using the hundred, which has an average area of approximately 118 square miles, as the basic territorial unit. Seasonal weather was represented by the rains in three subdivisions of the growing period of the crop, and these were taken as independent variates in an analysis by multiple regression. The regression of yield on rainfall, when considered as a function of time, takes a mathematically simple parabolic form with a maximum in winter and zeros in autumn and spring or early summer, and the coefficients obtained provide a clear demonstration of the sub-optimal character of average seasonal rainfall over the greater part of the season and almost throughout the wheat belt. The conclusions drawn from the general survey are substantiated by analyses of a limited number of exact records.

I. INTRODUCTION

In the Australian wheat belt the large fluctuations in yield are known to be dependent, to a very considerable extent, upon variations in the weather conditions prevailing in different years. Seasonal rainfall and its distribution in time constitute major determinants of yield, and associated factors, such as temperature and evaporation, also play their parts by modifying the duration of the season and determining the effective rainfall available to the crop. From accumulated experience, and recognition of the fact that the effects of any one meteorological element, for example rainfall, must vary throughout the season, the concept of an ideal distribution to meet the exigencies of critical periods during crop growth, has been developed. Apart, however, from such broad generalizations very little is known regarding the quantitative relationships of this phase of agricultural meteorology, notwithstanding the importance of such knowledge from both the scientific and the industrial points of view, including in the latter the direct and indirect value of forecasts in crop insurance and to trade and administration. This almost entire lack of accurate information is due principally to the complexity of the problem involved in the specification of the weather itself and the extreme paucity of reliable data.

Up to the present time the principal studies of Australian conditions have been contributed by Perkins and Spafford (1911, 1915), Richardson (1923),

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Perkins (1924), and Barkley (1927); reference is made again to the work of these authors in Section IV. With the advent of more powerful statistical techniques and accumulation of further data, the opportunity has been taken to carry out additional investigations. In a recent paper (Cornish 1949), the temporal trends of wheat yields in South Australia were discussed. The period chosen for examination was 1896-1941 inclusive and the analysis extended to practically the entire wheat belt of the State, all major soil groups and variants of climatic conditions within the area being represented. As a preliminary to the evaluation of the trends it was necessary to estimate and to eliminate the effects of variations in seasonal rainfall on yield, and the paramount importance of this step, with respect to both principle and accuracy, is illustrated by the results established. After making due allowance for changes in the seasons, it was possible to show that, in the maze of unordered data from this extensive and heterogeneous area, yields followed courses which were classifiable into two sequences, each containing a few simple types intimately related to the nitrogen status of the soils. Districts possessing the several types of trend were accurately delineated and conflicting opinions regarding the maintenance of fertility under the cropping systems in use were clarified, particularly for those sections known as marginal lands. As an immediate consequence, it is now possible from consideration of the trends and relevant observations on the soils and rainfall, including reliability of the latter, to decide upon the nature of remedial measures, their urgency and chances of success in individual districts.

In the paper cited no consideration was given to the effects of rainfall and one of the present main objectives is to take up this point. Since, for reasons which will be advanced later, the analysis must be regarded as only a general survey, a limited number of exact records obtained from experimental farms and private individuals have also been examined with a view to providing substantiation of the conclusions drawn from the general study.

II. THE GENERAL SURVEY

(a) *Data and Analytical Method*

The data and analytical technique that were employed have already been described (Cornish loc. cit.) so that only the salient features need be repeated here.

(i) *Yield Data*.—The smallest territorial unit for which consecutive yield data are available is the hundred, the mean area of which in South Australia is approximately 76,000 acres, or 118 square miles. This unit was adopted and the yields, expressed as mean yields in bushels per acre *sown for grain* were extracted from the South Australian Statistical Register for all hundreds with an average area of not less than 1,000 acres under crop. The yields used in compiling the records are not estimates but are derived from returns submitted by the growers. Figure 1 shows the distribution, within the regional divisions of the State (Regional Planning Committee 1946), of the 296 hundreds that were examined.

In districts where cropping began prior to 1896, the analysis was confined to the period 1896-1941 inclusive and where it commenced in 1896 or later only those hundreds which by 1941 had records of 20 years or more were selected. These limitations were imposed for three reasons:

1. All records of yield prior to 1896 are incomplete owing to the occurrence of two gaps, totalling seven years, when no returns were taken during 1885-88 and 1893-95, and sections of the records which exist do not give yields in sufficient detail for the purposes of the analysis.
2. In 1942 the Commonwealth Government under war-time legislation, restricted the acreage to be sown to wheat.
3. The minimum of 20 years was chosen so that the trend in yield and the effects of seasonal rainfall could be accurately assessed.

(ii) *Rainfall Data*.—The finest subdivision of the year for which rainfall data were available in a form convenient for immediate use was the calendar month. This unit was taken and the data supplied by the Commonwealth Meteorological Bureau.

As so much was contingent upon making proper allowance for seasonal variations in yield, it was necessary to select the rainfall stations carefully. Owing to the circumstances that the area of each hundred is comparatively large, and that in many instances, particularly those hundreds on the slopes of the Mt. Lofty and Flinders Ranges, there are considerable changes in altitude, the choice of rainfall stations would have been very difficult and of doubtful value, had recourse not been taken to two subsidiary sets of information:

1. A map showing the location of the area under crop in each hundred.
2. A map of isohyets of April-November rainfall.

In the majority of hundreds only one rainfall record was used, particularly in the regions Nuyts, Eyre, Pyap, and Pinnaroo, where rainfall changes comparatively slowly with position; occasionally there were two and more rarely three. In all, some 261 records were used to estimate the effects of seasonal rainfall.

(iii) *The Choice of Rainfall Variates*.—The choice of rainfall variates was made after consideration of the following factors: (1) The nature and paucity of the yield and rainfall data; (2) the average distribution of seasonal rainfall; (3) the form of a previously determined regression function; (4) the period during which rainfall is effective; and (5) the general insignificance of the regressions of yield on November rainfall as determined in a previous analysis; and fell on the rains of the subdivisions:

April and May
June, July, and August
September and October,

as suitable variates in effectively allowing for variations in seasonal conditions.

It is possible that rainfall of the preceding season and of the period December-March immediately prior to seeding are correlated with yield but

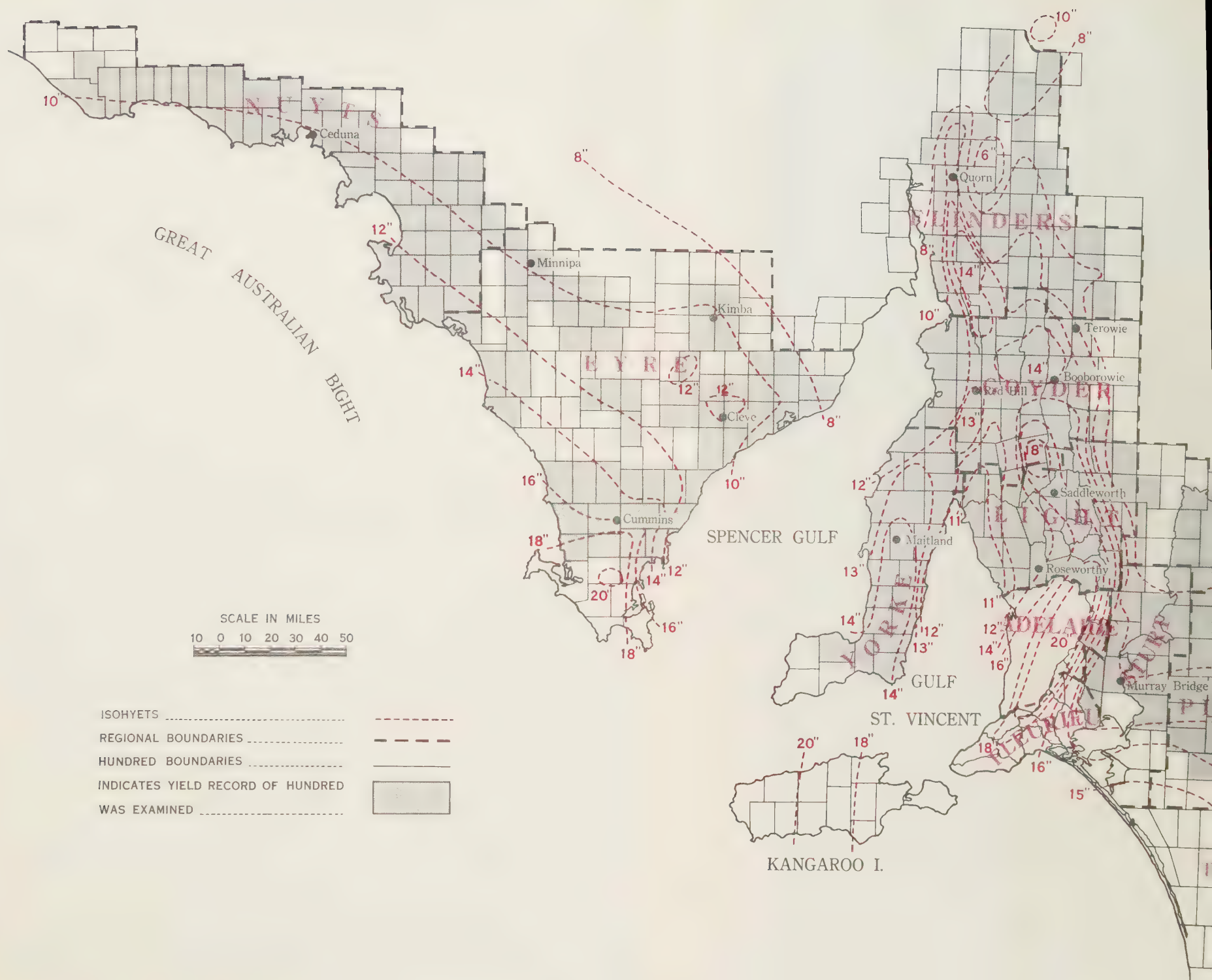


Fig. 1. – The wheat belt of South Australia.

such effects, if they exist, are small compared with those of the current season's rainfall to the end of October, and consequently they have been ignored. In any case the crop records are not of sufficient length to account for them adequately. Finally it is obvious that definite optimal conditions must apply in various parts of the season and the effects of rainfall at all times are not strictly independent and additive. In the present enquiry the quadratic terms corresponding to such factors have also been omitted, since in comparison with the linear terms they are of much less quantitative importance.

(iv) *The Statistical Technique.*—The statistical technique was that of multiple regression. Four different functions of time were employed to represent the temporal trends of yield, and if y denotes yield, x_1, x_2, x_3 the rainfall variates in the order given above, x_4 and x_5 time and its square, the types of multiple regression were

$$Y = \bar{y} + b_1(x_1 - \bar{x}_1) + b_2(x_2 - \bar{x}_2) + b_3(x_3 - \bar{x}_3) + b_4(x_4 - \bar{x}_4) \dots (1)$$

$$Y = \bar{y} + b_1(x_1 - \bar{x}_1) + b_2(x_2 - \bar{x}_2) + b_3(x_3 - \bar{x}_3) + b_4(x_4 - \bar{x}_4) + b_5(x_5 - \bar{x}_5) \dots (2)$$

$$Y = b_1(x_1 - \bar{x}_1) + b_2(x_2 - \bar{x}_2) + b_3(x_3 - \bar{x}_3) + b_4x_4b_5 \dots (3)$$

$$Y = b_1(x_1 - \bar{x}_1) + b_2(x_2 - \bar{x}_2) + b_3(x_3 - \bar{x}_3) + x_4/(b_4 + b_5x_4), \dots (4)$$

the bar over a symbol designating the arithmetic mean.

For all cases in which regressions of types (1) and (2) were used, the multiple correlation coefficient was determined and thence the percentage of variance of yield, A , ascribable to the average effects of the rainfall variates and time, from the relation

$$A = 1 - \frac{(n-1)}{(n-p-1)} (1 - R^2),$$

where R is the multiple correlation coefficient, n is the number of observations in the sample, and p the number of independent variates (Fisher 1924). Where regressions of types (3) and (4) were fitted an index of multiple correlation was calculated as the ratio

$$R^2_I = \frac{2SyY - SY^2 - n\bar{y}^2}{S(y - \bar{y})^2},$$

and the percentage of variance from the relation

$$A_I = 1 - \frac{(n-1)}{(n-p-1)} (1 - R^2_I),$$

p now designating the number of coefficients in the regression formula after deducting one for the mean.

(b) *Characteristics of the Season*

The most prominent feature of the season is the marked winter incidence of the rains. Approximately 70-80 per cent. of the annual precipitation occurs within the period April-October inclusive, the summer months being charac-

terized by hot, dry atmospheric conditions, low rainfall, and high evaporation. Within the wheat belt, seasonal rains vary over the rather wide range 6 in. to 18 in. and their geographical distribution is illustrated by the isohyets of Figure 1.

Further details are presented in Table 1. Twelve stations, of which the locations are given in Figure 1, have been chosen as representative of the conditions prevailing in different parts of the wheat belt and the quantities tabled all relate to the rainfall variates used in the multiple regression equations. With all three variates the standard deviations per annum, in absolute terms, increase very closely in direct proportion to the means. Relative to the means April-May (breaking) rains and September-October (spring) rains are more variable than June-July-August (winter) rains; the first two show a general tendency to decrease as the mean increases, the effect being slightly more pronounced for April-May rains. Apart from the two most variable stations (Quorn and Loxton), the relative variability of the winter rains is comparatively constant. In all rainfall variates, the annual variation is very great, particularly when it is recalled that the variates are the total rains occurring in periods of 2 or 3 successive months; if shorter periods had been considered the relative variability would have been very much greater.

TABLE 1
CHARACTERISTICS OF SEASONAL RAINFALL

Station	Length of Record (years)	Rainfall Variates (in.)						Mean (Apr.-Oct.) (in.)	Intra-station Correlations		
		x_1		x_2		x_3			x_1x_2	x_1x_3	x_2x_3
		Mean	Stand. Dev.	Mean	Stand. Dev.	Mean	Stand. Dev.				
Ceduna	44	2.09	1.13	4.53	1.57	1.75	0.79	8.37	0.18	0.00	-0.02
Cleve	46	2.59	1.36	5.54	1.66	3.03	1.41	11.16	0.12	0.02	0.11
Minnipa	26	2.03	0.96	5.66	2.10	2.41	1.21	10.10	0.33	-0.03	0.13
Cummins	35	2.94	1.25	8.42	2.71	3.26	1.38	14.62	0.23	0.08	0.10
Terowie	46	1.84	1.09	4.43	1.63	2.55	1.28	8.82	0.07	0.01	0.33
Quorn	46	1.69	1.33	4.61	2.51	2.25	1.30	8.55	0.11	0.07	0.24
Red Hill	46	2.55	1.25	5.70	1.96	2.78	1.35	11.03	0.29	0.16	0.35
Loxton	46	1.58	1.03	3.23	1.43	2.24	1.41	7.05	-0.02	-0.16	0.20
Saddleworth	46	3.75	1.51	7.04	2.25	3.93	1.64	14.72	0.37	-0.01	0.19
Maitland	46	3.85	1.53	7.75	2.67	3.57	1.46	15.17	0.18	0.18	0.22
Murray Bridge	46	2.34	1.17	4.35	1.53	2.71	1.24	9.40	0.17	0.18	0.17
Bordertown	46	3.32	1.40	6.66	2.04	3.80	1.46	13.78	0.10	-0.06	0.21

Table 1 also gives the intra-station correlations between the three variates. Four of the 36 coefficients show evidence of weak covariation but a test of association, derived from the inverse hyperbolic transformations of the correlations, $z = \tanh^{-1}r$, gives $\chi^2 = 49.20$ with 36 degrees of freedom for which $0.05 < P < 0.10$, showing that the rains in the several parts of the season are practically independent, as judged from the data as a whole.

TABLE 2
INTER-STATION CORRELATIONS

	Cleve	Minnipa	Cummins	Terowie	Quorn	Red Hill	Loxton	Saddlesworth	Maitland	Murray Bridge	Bordertown
					Variate x_1						
Ceduna	0.42	0.72	0.48	0.56	0.62	0.60	0.52	0.54	0.50	0.52	0.32
Cleve		0.69	0.63	0.42	0.45	0.63	0.44	0.45	0.63	0.37	0.47
Minnipa			0.53	0.41	0.50	0.55	0.48	0.61	0.61	0.12 (n.s.)	0.51
Cummins				0.56	0.37	0.55	0.54	0.60	0.67	0.34	0.65
Terowie					0.73	0.74	0.85	0.74	0.57	0.52	0.53
Quorn						0.77	0.67	0.58	0.59	0.58	0.37
Red Hill							0.60	0.69	0.72	0.49	0.46
Loxton								0.67	0.47	0.54	0.63
Saddlesworth									0.80	0.56	0.64
Maitland										0.61	0.61
Murray Bridge											0.41
					Variate x_2						
Ceduna	0.58	0.74	0.63	0.61	0.52	0.60	0.46	0.57	0.62	0.59	0.51
Cleve		0.80	0.73	0.67	0.55	0.60	0.56	0.65	0.67	0.66	0.66
Minnipa			0.92	0.85	0.81	0.81	0.67	0.79	0.79	0.62	0.69
Cummins				0.85	0.79	0.83	0.71	0.80	0.85	0.74	0.80
Terowie					0.88	0.86	0.74	0.73	0.76	0.76	0.71
Quorn						0.83	0.77	0.68	0.73	0.64	0.62
Red Hill							0.81	0.80	0.88	0.72	0.65
Loxton								0.78	0.74	0.65	0.70
Saddlesworth									0.81	0.63	0.79
Maitland										0.66	0.75
Murray Bridge											0.64
					Variate x_3						
Ceduna	0.57	0.86	0.46	0.46	0.51	0.44	0.38	0.41	0.41	0.49	0.28 (n.s.)
Cleve		0.83	0.57	0.67	0.66	0.77	0.76	0.77	0.67	0.77	0.55
Minnipa			0.70	0.75	0.67	0.72	0.67	0.67	0.56	0.72	0.54
Cummins				0.65	0.47	0.71	0.52	0.76	0.67	0.68	0.66
Terowie					0.85	0.82	0.76	0.73	0.57	0.69	0.65
Quorn						0.75	0.72	0.54	0.42	0.52	0.42
Red Hill							0.72	0.85	0.79	0.70	0.69
Loxton								0.69	0.54	0.71	0.62
Saddlesworth									0.88	0.75	0.83
Maitland										0.67	0.78
Murray Bridge											0.64

All coefficients are statistically significant (minimum odds 19 : 1) except the two indicated.

The inter-station correlations of Table 2 are in marked contrast, and indicate the very widespread nature of the disturbances producing the rains, despite the fact that the correlations decrease as inter-station distance increases. There is thus a definite tendency for the simultaneous occurrence of either very wet or very dry conditions over the whole wheat belt.

(c) Discussion of Results

The complete tabulation of the regressions of yield on rainfall has not been included since it is too extensive for reproduction herein; but all regressions are presented in summary tables and some of them graphically. Copies of the original data have, however, been filed and are available for inspection.*

Before proceeding to discussion of the results, it must be emphasized that only one meteorological element has been used to characterize the season, so that, in correlating it with yield, the regression obtained is the resultant of a number of components, one the direct effect of the rainfall *per se*, and the remainder due to the direct and indirect effects of all other elements to the extent to which they are associated with the rainfall occurring in the several subdivisions under consideration.

TABLE 3
DISTRIBUTION OF COEFFICIENTS AND INDICES OF MULTIPLE CORRELATION WITHIN
ARBITRARY CLASSES OF SEASONAL RAINFALL

Correlation	Rainfall (in.)							Total	Per Cent. Variance (A)
	< 8	8-10	10-12	12-14	14-16	16-18	> 18		
≥ 0.91	7	2	2	3	—	—	—	14	83
0.81-0.90	39	54	44	29	3	—	—	169	70
0.71-0.80	9	34	19	9	9	3	1	84	53
0.61-0.70	—	3	12	4	1	1	—	21	37
0.51-0.60	—	2	1	2	—	1	—	6	25
0.41-0.50	—	—	—	2	—	—	—	2	14
Total	55	95	78	49	13	5	1	296	

Table 3 sets out the distribution of the coefficients and indices of multiple correlation within arbitrary but convenient subdivisions of the range of seasonal rainfall and the mean values of the percentage variance, the value 83 per cent., for example, being the mean of the 14 cases in which the coefficient or index was ≥ 0.91 . In 267 hundreds, 50 per cent. or more of the observed variation in yield is expressible in terms of the rainfall variates and time, a fact sufficient to excite remark when it is recalled that all other causes of variation without exception are included in the remaining 50 per cent. or less. The

* At the libraries of the Commonwealth Scientific and Industrial Research Organization, 314 Albert Street, East Melbourne, and its Section of Mathematical Statistics, University of Adelaide.

relation which exists between the measures of correlation and seasonal rainfall reflects largely the strength of the relation between yield and rainfall, since in the majority of hundreds the regression of yield on time is the smaller contributor to the total variation ascribable to the regression formula. Considering the results as a whole, it can be claimed that a rainfall record provides a sufficiently accurate index of seasonal conditions in this environment.

Table 4 lists the distribution of the correlations within the several soil types of the wheat belt.* As rainfall and soil type are to some extent related, these distributions are not completely independent of those in Table 3, but nevertheless they do indicate that the claim made above holds also on all principal soils. The grand total of 335 does not agree with that of Table 3 because 39 hundreds, comprised equally of two soil types, have been included under both in preparing the table.

TABLE 4
DISTRIBUTION OF COEFFICIENTS AND INDICES OF MULTIPLE CORRELATION
WITHIN SOIL TYPES

Correlation	Soil Type											Total
	Mallee Types					Desert Loam	Solonetz	Red Brown Earth	Rendzina	Podsol		
	Sandy	Stony	Loamy	Mixed	Transitional						Calcareous Aeolianite	
≥ 0.91	2	4	3	3	—	—	3	—	2	—	—	17
0.81-0.90	39	10	27	19	6	15	28	1	34	6	1	186
0.71-0.80	16	8	9	5	15	7	15	2	13	2	5	97
0.61-0.70	2	3	4	—	9	1	1	1	1	3	1	26
0.51-0.60	—	1	—	—	2	—	1	1	—	—	1	6
0.41-0.50	—	—	1	—	1	—	—	—	—	1	—	3
Total	59	26	44	27	33	23	48	5	50	12	8	335

This claim is illustrated from a complementary viewpoint by the frequency distributions of the hundreds and significant regressions in the double classification of soil type and rainfall in Table 5, in which allocations to the soil groups are the same as those used in the construction of Table 4. With the exception of the minor soil groups, solonetz, rendzina, and podsol, which represent a very small fraction of the total area, all soils possess high proportions of significant regressions over their respective ranges of rainfall. The coefficients b_2 are outstanding and nearly all are significant. Approximately 50 per cent. of each of b_1 and b_3 reach significance, but with these regressions the proportions fall rather more rapidly than with b_2 as the value of the corresponding rainfall variate increases.

* General descriptions of the soil types were given by the author (Cornish loc. cit.) and greater detail is obtainable from numerous publications of C.S.I.R.O.

Data for the principal soils are presented graphically in Figures 2-6 inclusive.

Red Brown Earths.—The three diagrams of Figure 2 show clearly that the magnitudes of the regressions decrease as the mean values of the rainfall variates increase, and as far as can be judged from the data these relationships are linear. Broadly speaking, hundreds classed in this soil group constitute a

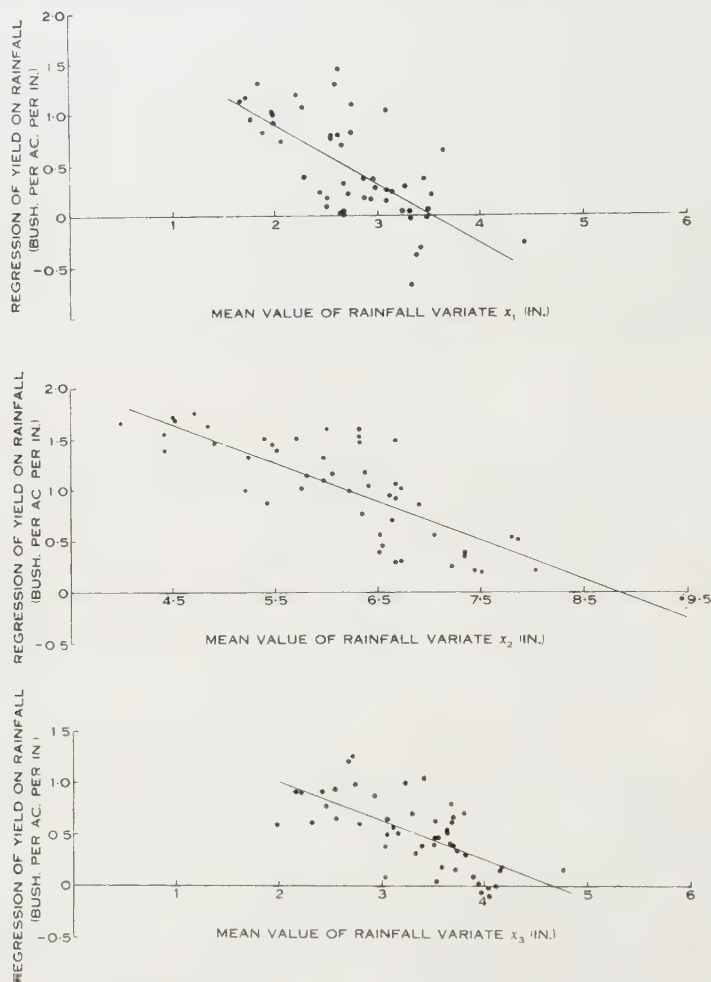


Fig. 2.—Regression coefficients of the red brown earths in relation to the corresponding rainfall variates.

homogeneous set with respect to the cropping methods in use and consequently the gradual fall in the regressions can be taken as evidence that the corresponding rainfall variates are approaching optimal values. Further reference is made to this point below.

Loamy Mallee.—Regression coefficients of the hundreds allocated to this soil type are plotted in Figure 3 and the diagrams indicate an association with

rainfall. With respect to b_1 and b_2 in particular the relationships are weakened to some extent by a subdivision of the points which depends partly on a climatic differentiation and partly on a difference in standards of agriculture, according to regional location of the hundreds in (a) Eyre, and (b) Goyder, Yorke, and Light. To obtain rough approximations to the optimal rains, heterogeneity due to different standards and climate have been ignored and the lines have been fitted using each complete set of observations.

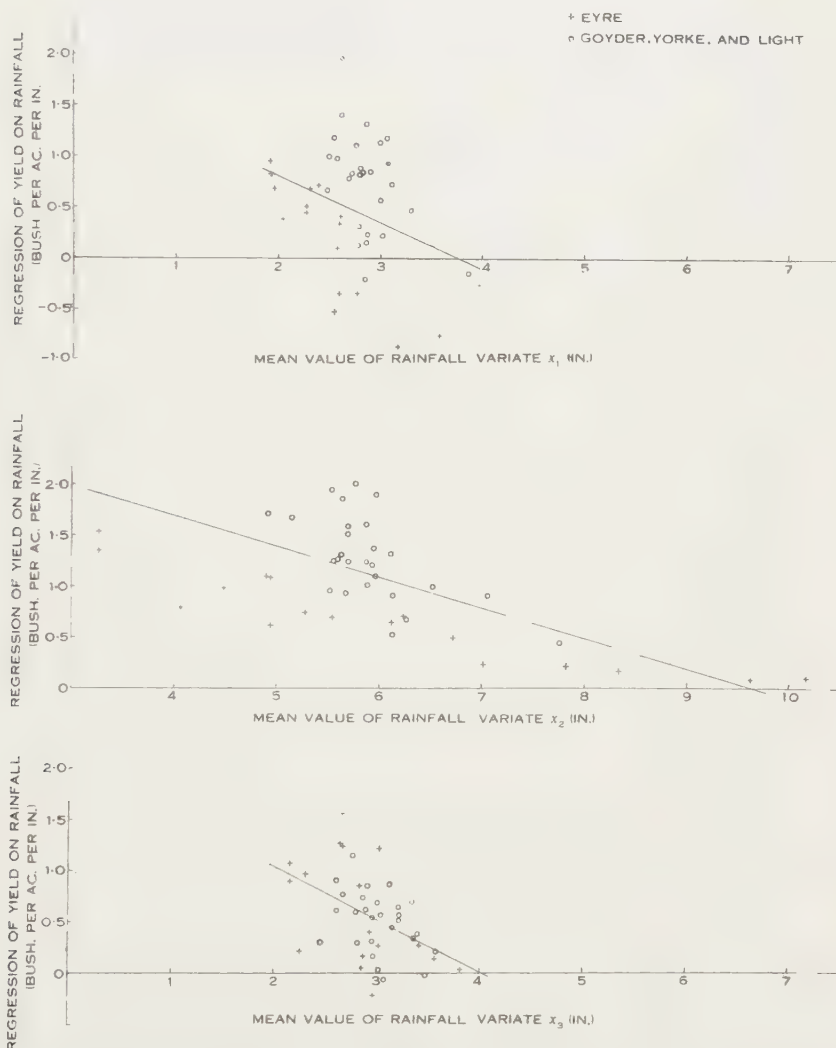


Fig. 3.—Regression coefficients of loamy mallee soils in relation to the corresponding rainfall variates.

Sandy Mallee.—Figure 4 shows the scatter diagrams of this soil type. The coefficients fall into two fairly distinct groups, the differentiation again being clearer for b_1 and b_2 than for b_3 . In this case the regional subdivisions are

(a) Nuyts and Eyre, and (b) Flinders, Goyder, Yorke, Light, Pyap, and Pinnaroo. There is no evidence that either b_1 or b_3 decreases as the corresponding rainfall variate increases; b_2 falls slightly but consideration of the data indicates that this is not due to the fact that rainfall is approaching an optimal value. Nearly all coefficients with values less than $1\frac{1}{2}$ bushels per acre per inch and lying to the right of the diagram are from hundreds which possess low values of b_1 .

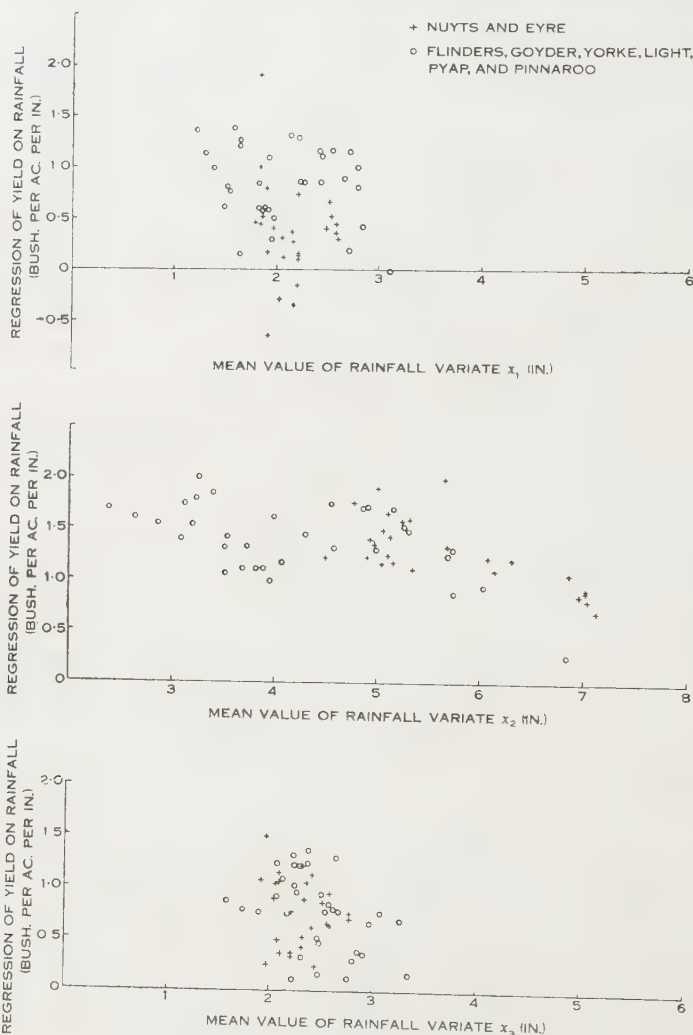


Fig. 4.—Regression coefficients of sandy mallee soils in relation to the corresponding rainfall variates.

Stony Mallee and Mixed Mallee.—In these two types the diagrams resemble those of the sandy mallee soils, but dispersion of the points is not so great and there is no evidence of grouping according to regional location of the hundreds.

Transitional Mallee-Solonetz.—This group occurs in two widely separated regions, Pinnaroo and Eyre, and it is clear in Figure 5 that no differentiation exists on this basis. The regressions are not related to rainfall.

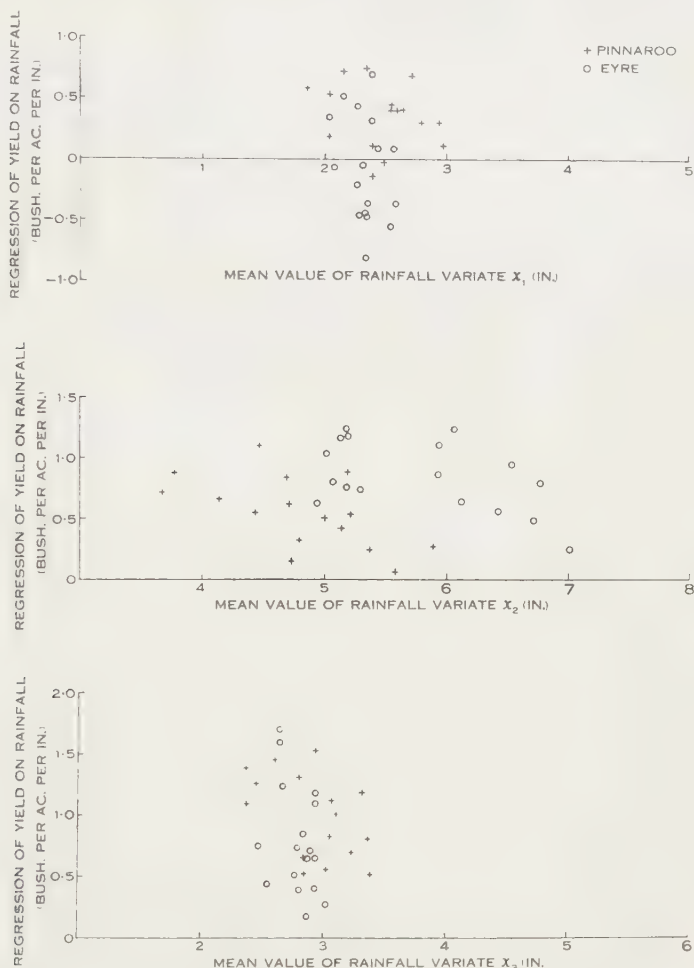


Fig. 5.—Regression coefficients of transitional mallee-solonetz soils in relation to the corresponding rainfall variates.

Calcareous Aeolianite.—The diagrams are similar to those in Figure 5.

Desert Loams.—Figure 6 illustrates the position in this soil type. The grouping of the coefficients according to location of the hundreds in (a) Nuyts, and (b) Flinders and Eyre is very distinct, but there is no sign that the rains approach optimal values.

The data of the red brown earths and loamy mallee soils have been fitted with weighted regression lines but it has been assumed that the coefficients within each set are independent. Obviously this condition is not fulfilled. Each set of coefficients is distributed approximately in a multivariate normal

distribution with an unknown matrix of variances and covariances. As, however, the principal object of the analysis was to make an empirical fit of the lines, tests of significance being of secondary interest only, statistical dependence of the data is of no great consequence.

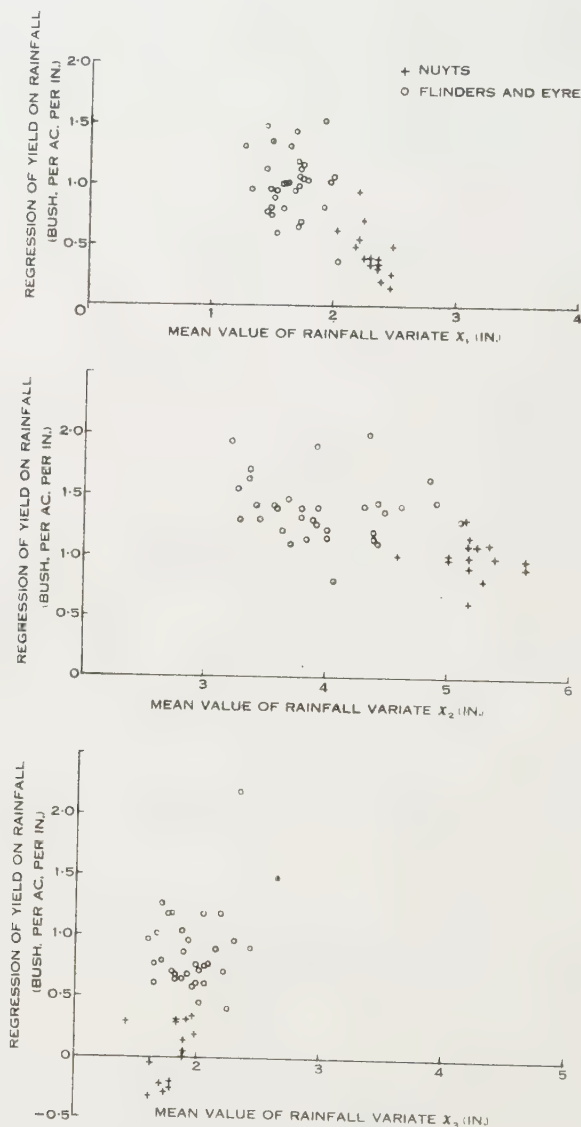


Fig. 6.—Regression coefficients of the desert loams in relation to the corresponding rainfall variates.

The frequency distributions of the significant regressions in Table 5 and the evident preponderance of high positive values, as indicated in the figures, provide a clear demonstration of the sub-optimal character of average seasonal rainfall over the greater part of the season and almost throughout the wheat belt.

TABLE 5
FREQUENCY DISTRIBUTIONS OF SIGNIFICANT REGRESSION COEFFICIENTS WITHIN
SOIL TYPE — RAINFALL CLASSES

		Mallee Types										Soil Type					Total	
		Sandy	Stony	Loamy	Mixed	Transitional	Calcareous	Aeolianite	Desert Loam	Solonetz	Red Brown Earth	Rendzina	Podsol					
Coefficient	Rainfall (in.)	27	13	11	3	2	15	15	1	—	5	1	32	31	1	—	8	81
	1-2	31	12	15	5	32	15	12	4	32	2	18	10	16	2	—	25	189
	2-3	1	—	—	9	4	—	—	—	—	—	—	—	—	4	—	16	61
	3-4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	8
	4-5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	2
Total		59	25	26	16	44	21	27	19	33	2	23	11	48	33	5	50	335
b ₂	2-3	3	3	2	2	—	—	2	2	—	—	—	—	21	21	1	1	7
	3-4	15	15	8	8	2	2	5	5	2	2	—	—	14	14	1	1	55
	4-5	12	12	6	6	6	6	14	14	9	7	8	8	13	13	—	7	76
	5-6	18	18	4	3	21	21	5	5	15	12	14	14	13	13	1	12	98
	6-7	7	6	5	4	8	8	1	1	6	5	—	—	—	—	3	20	55
	7-8	4	4	1	1	4	2	—	—	1	—	1	1	—	—	—	8	43
	8-9	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	4	25
	9-10	—	—	—	—	1	—	—	—	—	—	—	—	—	—	1	5	
	10-11	—	—	—	—	1	—	—	—	—	—	—	—	—	—	1	—	6
	Total	59	58	26	24	44	39	27	27	33	26	23	23	48	48	5	50	335
b ₃	1-2	6	4	3	3	—	—	2	2	—	—	13	3	34	18	—	1	293
	2-3	50	29	18	12	26	11	21	18	24	14	10	7	14	11	1	12	59
	3-4	3	2	5	2	18	3	4	2	9	8	—	—	—	—	4	31	182
	4-5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6	88
	Total	59	35	26	17	44	14	27	22	33	22	23	10	48	29	5	50	335
Total		59	35	26	17	44	14	27	22	33	22	23	10	48	29	5	50	335
Total		59	35	26	17	44	14	27	22	33	22	23	10	48	29	5	50	335

Frequencies of significant coefficients are printed in italics.

The diagrams also show that the response to rainfall varies among the soil types as well as within the types to which reference has been made, but before making further comment it must be emphasized that this study is, by nature, only a broad survey. Obviously with respect to any particular hundred there is on the one hand a set of factors tending to increase the responses to rainfall and on the other a set tending to decrease them. The observed regression on any rainfall variate is the resultant of their effects, and because of the nature of the data they are completely confounded and incapable of separate assessment; in fact, it is not possible to enumerate either set exhaustively. In consequence the labour involved in providing greater detail than that presented by a series of mean values is not warranted, and it would be unreasonable to give more than a few general observations. It may be anticipated, however, that the accuracy of comparisons between the means will be enhanced since the regressions have been derived either from the data of identical sequences of seasons or from series having a large proportion of years in common.

TABLE 6
STANDARDIZED REGRESSIONS AND STANDARD DEVIATIONS OF RAINFALL VARIATES
AND YIELD

Soil Type	b'_1	s_1	b'_2	s_2	b'_3	s_3	s_y
Sandy mallee (a)	0.47	1.10	1.12	1.64	0.52	1.29	2.04
(b)	0.15	1.09	1.07	2.05	0.39	1.21	2.45
Stony mallee	0.40	1.06	0.97	1.67	0.48	1.25	1.87
Loamy mallee (a)	0.35	1.30	0.92	1.99	0.24	1.32	2.72
(b)	0.07	1.26	0.47	2.07	0.30	1.29	2.53
Mixed mallee	0.37	1.10	0.96	1.68	0.58	1.28	2.27
Transitional mallee-solonetz	0.08	1.14	0.57	1.89	0.55	1.33	2.20
Calcareous aeolianite	0.32	1.08	1.13	1.85	0.32	0.96	1.96
Desert loam (a)	0.56	1.22	1.20	1.95	0.47	1.09	2.15
(b)	0.25	1.26	1.00	1.97	0.03	0.90	1.99
Red brown earth	0.21	1.37	0.70	2.16	0.26	1.53	3.07

The subdivisions of sandy mallee, loamy mallee, and desert loam correspond to those given in the text.

In absolute terms, the residual standard deviation of yield and the standard deviations of the rainfall variates vary considerably, and consequently to render the regression coefficients directly comparable, it is necessary to standardize them by multiplying each by the ratio

$$\frac{\text{standard deviation of rainfall}}{\text{standard deviation of yield}}$$

Average values of these standardized regressions, denoted by b'_i , are set out in Table 6 together with the corresponding average standard deviations of yield, s_y , and the rainfall variates, s_i , to facilitate reversion of the regressions to their original dimensions. The figures given are averages of all hundreds classified in the several soil types listed in the table.

With respect to any particular regression the contrasts are now substantially reduced, but several still persist. Transitional mallee-solonetz behaves differently from all other soils and the differences between the subdivisions of the sandy mallee and desert loam types stand out clearly. Seasonal rains vary in the neighbourhood of optimal values throughout considerable areas of the red brown earths and loamy mallee soils and consequently the means of these two groups are lower than those of many of the remaining soil classes. Within the latter set, the means of all three coefficients are lower than might be expected on the basis of a comparison with the corresponding quantities from those parts of the red brown earths and loamy mallees with a comparable rainfall, the differences observed being attributable to a number of factors which may be integrated appropriately in the term "standard of wheat farming." In varying degree, the yields of all mallee districts have been affected by the lower standard of the practices adopted. The majority of hundreds in these areas were opened for cultivation after 1896, and consequently the pioneering phase represents an increasingly larger proportion of the period under review as the date of settlement advances from 1896. Cropping methods in the early stages are exploitative through circumstances incidental to pioneering on land carrying mallee vegetation. Mallee stumps remaining in the ground after the first clearing repeatedly send out new growth and the settler crops continuously for some years, burning the stubbles to destroy the shoots. During this phase, which may take up to 10 years to complete, the principal factor limiting yield, apart from rainfall, is probably a deficiency of nitrogen. In the course of time, yields improve as fallowing and other measures for increasing production are introduced, but the methods of cropping employed do not reach the same standard as in districts with a rather more reliable rainfall. Under such conditions the response of the crop to rainfall is naturally not as great as it would otherwise be.

The most significant observation is, however, that the figures quoted establish the greater effectiveness of winter rains as compared with autumn and spring rains; on all soils, apart from transitional mallee-solonetz, b'_2 is 2-2½ times as great as either b'_1 or b'_3 . In the past considerable emphasis has been laid upon the importance of spring rains, but it is clear from the sign, magnitude, and duration of the response that the ultimate success of the harvest depends upon favourable rains throughout the season, and in particular during the winter months. Seeding operations, when properly conducted, commence after the opening rains and range from mid-April to the end of June, depending upon date of break in the season and location within the wheat belt. The mean time of seeding can be taken as the last week of May and it is significant that rain above average for some weeks prior to sowing should have a marked influence on yield. Early rains facilitate preparations for seeding and coincide with soil temperatures that are still high enough to promote germination and early development, two factors of material value in a growing season of short duration. The characteristic feature of autumnal weather over a large proportion of the South Australian wheat belt is that suitable

conditions for seeding operations, rapid germination, and early growth, are confined to a very limited period. Earlier seeding is hazardous and later seeding almost invariably leads to unsatisfactory development which can only be successful in the event of a protracted and otherwise favourable season.

As time advances from April 1, average effective rainfall increases as a result of the combined action of increasing rainfall and associated atmospheric conditions of rising humidity, decreasing temperature, and decreasing solar radiation. The increasing response to winter rains is due principally to an indirect effect following upon accumulation of water reserves for use in later stages of the life cycle, but there exists also a direct effect in stimulating surface growth since temperatures do not fall to the point where development is arrested completely.

From mid-August, growth and transpiration accelerate rapidly as each atmospheric condition traverses the second part of the course in its periodic movement. The fall in response to rainfall in the latter portion of the season can reasonably be ascribed to the interplay of several factors. In the first place, average effective rainfall is rapidly reduced as the season advances and the regression shows, in reality, the benefit from only the available moisture. Secondly, rain during September, October, and November is often preceded by strong north winds which directly damage the crop through shattering, lodging, etc., and finally, if suitable conditions prevail after rain, they are conducive to the development of disease, for example rust.

The regularity and simplicity of the changes in magnitude of the coefficients, taken in their natural order, suggest that the regression of yield on rainfall, considered as a function of time, assumes a form of simple character, which could, in fact, be represented accurately by a parabola having a maximum in winter and zeros in autumn and spring or early summer. Later sections will demonstrate that this actually holds true over a large proportion of the wheat belt. The shape of the curve would be expected to vary with soil, standard of cropping, stage of development, and so on but the effects of these factors are subordinate to the dominating influence of rainfall. In regions of lowest rainfall, the curve has its greatest maximum, but as the mean rainfall increases, the winter maximum is reduced in value, accompanied by a general flattening of the curve. An extremely simplified diagrammatic representation is given in Figure 7, where the regression functions, in order, correspond to districts with increasing mean rainfall.

The change in the form of these curves, as the mean rainfall increases, is readily explicable when consideration is given to the concept of optimal rains. Thus, corresponding to any particular time t during the season, there will be an optimal rainfall, and the relation between yield and rainfall at t would be expected to follow a curve rising to, and falling from, the yield at the optimum, in a manner resembling that given in Figure 8; as drawn the curve is a parabola but there are no theoretical grounds to suggest that the true relationship would possess this particular mathematical form. The four points marked on the abscissa in Figure 8 correspond to the mean rainfall at t in the

four districts of Figure 7 and constitute a sequence of values approaching optimal rain. Tangents at the points where the four ordinates intersect the curve represent a series of diminishing positive regression coefficients corresponding to the ordinates at t of the four curves in Figure 7.

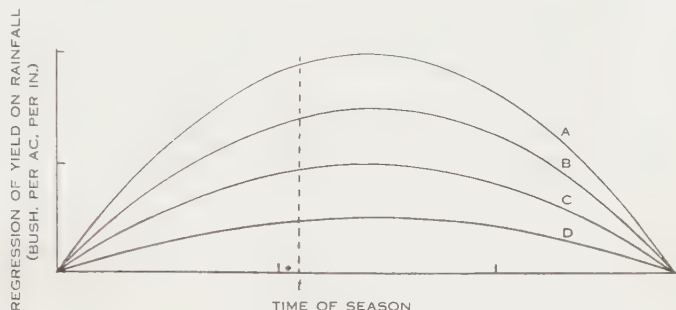


Fig. 7.—Diagrammatic representation of regression functions in four districts, A, B, C, and D in order of increasing rainfall.

It was expected originally that direct evidence of well-defined optimal conditions would be obtained for individual hundreds throughout a substantial proportion of the wheat belt, but as mentioned previously, an exhaustive examination failed to reveal, in any instance, corresponding quadratic terms of quantitative importance, the greater part of the effect of rainfall being expressible

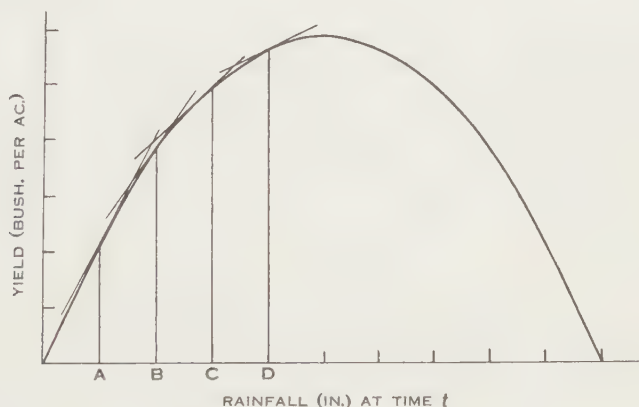


Fig. 8.—Relation between yield and rainfall at time t , showing regression of yield on rainfall at t , in the four districts of Figure 7.

in terms of linear relations in the variates employed herein. The explanation of this feature lies simply in the fact that the rains actually received in the majority of seasons in the period under review are distributed over a range with an upper limit which is in defect of the optimum.

It is, however, possible with the red brown earths and loamy mallee types to make an indirect assessment of optimal rains by taking in Figures 2 and 3

the values where the fitted regression lines intersect the axes of the rainfall variates, and the relationship between yield and rainfall may be inferred from the solution of the elementary differential equation

$$dy/dx = a + bx,$$

where y denotes yield, x any rainfall variate, and a and b the constants of the appropriate regression line in the figures. Integration gives

$$y = k + ax + \frac{1}{2}bx^2,$$

and since the regressions are approximately linear, a parabola describes, with sufficient accuracy, the relation between yield and rainfall for the three subdivisions of the season used in the analysis. This convenient procedure has been adopted since it does not seem justifiable to press refinement too far owing to the nature of the data and the assumptions that have already been made. The equations to the curves for the red brown earths are as follows:

Rains of the Period	Equation	Optimal Rain (in.)
April-May	$y = k + 2.07x_1 - 0.29x_1^2$	3.6
June-July-August	$y = k + 3.39x_2 - 0.19x_2^2$	8.8
September-October	$y = k + 1.77x_3 - 0.19x_3^2$	4.7

so that optimal seasonal rains on this soil type amount to approximately 17 in. appropriately distributed. Ignoring regional differentiation, loamy mallee soils give the following results:

April-May	$y = k + 1.73x_1 - 0.23x_1^2$	3.8
June-July-August	$y = k + 2.90x_2 - 0.15x_2^2$	9.6
September-October	$y = k + 2.06x_3 - 0.26x_3^2$	4.0

yielding an optimal seasonal rain of nearly 17½ in., which, considering the crude nature of the estimation, agrees reasonably well with what would be expected in comparison with the red brown earths. The constant of integration must, of course, remain unknown in the absence of any knowledge regarding boundary conditions.

III. EXAMINATION OF INDIVIDUAL RECORDS

It is clear that the regressions for the yields of whole hundreds have been calculated on an extremely broad basis and for this reason an attempt has been made to substantiate the general results by examination of data obtained under more uniform conditions. Owing to the extreme difficulty of securing long and accurate records only a limited number were available; the locations of the places whence they were derived are given in Figure 1.

(a) Roseworthy

(i) *Yield and Rainfall Data.*—The yield data were taken from the records of the so-called permanent manurial experiment on wheat at Roseworthy Agricultural College. This trial was initiated in 1905 and designed on similar lines

to the classical experiment on Broadbalk at Rothamsted, but differed from the latter in two important features. At Rothamsted the manurial treatments were applied to single plots, but at Roseworthy the treatments were applied to pairs of plots, which for several treatments were replicated. Previous experience had demonstrated that it was essential to fallow and in order to accomplish this and simultaneously maintain the continuity of the record, each treatment was applied to two adjacent plots which were alternately cropped and fallowed. The trial was continued until 1930, by which time, notwithstanding the fallowing, the yields had declined to extremely low values. In 1931 all plots were fallowed but poor yields were obtained again in 1932, and for this and other reasons the trial was terminated.

In the original plan the experimental field comprised 122 acres divided into 61 two-acre plots. In 1911 a further 10 plots were added, making the area 142 acres. Over the major portion of the field the soil, which originally carried scattered groups of Murray box (*Eucalyptus largiflorens*), consists of a fairly uniform medium to heavy chocolate loam overlying a yellow-red clay subsoil and may be regarded as typically good wheat land. The only irregularity of note is due to small areas of travertine limestone with their covering of loose, light soil, characteristic of mallee country; soils of this type carry mallee (*E. dumosa* and *E. oleosa*) in their natural state. Each plot consisted of a narrow strip about $4/5$ chain in breadth and some 24-25 chains long. In 1905 the plot area was 1.97 acres but in subsequent years this was slightly reduced.

Owing to the numerous modifications and changes made in the original plan, the yields from only eight pairs of plots for the 26 seasons 1905-30 inclusive are suitable for analysis but two of these have been omitted since the manurial treatments are not now of any practical importance. The numbers and manurial treatments of the selected plots are given below:

Plot Number	Treatment
26 and 27	No manure
52 and 53	No manure
28 and 29	Superphosphate 2 cwt./ac.
58 and 59	Superphosphate 2 cwt./ac.
30 and 31	Superphosphate 2 cwt./ac., sodium nitrate 1 cwt./ac. applied at seeding
32 and 33	Superphosphate 2 cwt./ac., sodium nitrate 1 cwt./ac. applied in spring.

In 1909 sodium nitrate was not applied to plots 32-33 and the recorded changes in variety are as follows:

1905 Gluyas, 1906 King's Early, 1907 Gluyas, 1908 King's Early, 1909-22 King's White, 1924-27 Gluyas, 1928 Ford, 1929-30 Gluford.

The rainfall data were taken from the meteorological records at the College; rainfall was measured with a standard 8 in. gauge on a site approximately one mile from the experimental plots.

In 1923 excessive autumnal and early winter rains made normal seeding operations impracticable with the result that no crop yield is available for this year. Allowance for this fact has been made in the correlational analysis.

(ii) *Preliminary Analysis.*—It has been pointed out that yields were obtained from adjacent plots sown in alternate years. Inspection of the records showed clearly that the mean yields of the members of each selected pair were not significantly different but it was not obvious that the trends of these yields also differed only by errors of random sampling. Before proceeding with the main analysis it was deemed advisable, therefore, to subject the data to preliminary tests in order to assess the effect of the alternate fallows on the continuity of the records. The series of yields involving plots scheduled for sowing in 1923 were completed by inserting the average of the three preceding and three succeeding yields and the linear regression on time of each individual sequence determined. In all cases, the mean yields, linear regressions, and residual variances were not significantly different and consequently it was considered legitimate to combine the two series to form a single continuous record for each pair of plots.

(iii) *Analysis of Yield Data.*—On the assumption that the succession of seasons had been fortuitous, and this is justifiable, the linear regression of yield on time was determined and the total variation in yield partitioned into two components, the first ascribable to the regression and the second to annual fluctuations of yield about the fitted line.

Large and significant declines in yield were observed under all manurial treatments, and from the evidence available, they may be attributed primarily to increasing infestation of the plots with weeds, particularly wild oats (*Avena fatua*). Since the six unmanured plots averaged approximately 20 bushels per acre in 1905, there seems no doubt that, from the standpoint of local practice at the time, the experiment was initiated at a stage where the field was in a high state of fertility and it is possible that the fall in yield was due in part to depletion of this fertility, but it is certain that this factor is negligible in comparison with the predominant damaging effect of the weeds.

(iv) *Correlation of Yield and Rainfall.*—Fluctuations in yield about the course of the decline may be attributed to random variations in the seasons, and for the purpose of correlating them with the weather, the method developed by Fisher (loc. cit.) has been followed. Seasonal conditions have been represented conventionally by rainfall of the period March 1 to December 14, an interval of sufficient length to embrace all seasons in which rainfall is effective for some weeks prior to the average date of seeding (approximately May 30) and all seasons in which harvesting was delayed until early December.

The rainfall of this period in each year was divided into 48 six-day totals and to each set of values was fitted a series of orthogonal polynomial functions of the third degree in time, thus furnishing four constants, denoted by a' , b' , c' , and d' with which to express the quantity and distribution of rainfall in each season. As the linear component of secular change had been eliminated from each sequence of yields it was necessary to treat the rainfall distribution

values in a similar manner. Actually each series was fitted initially with a curve of the second degree in time. This precaution was taken because a previous analysis had demonstrated the presence of well-defined changes in the distribution of seasonal rainfall at Adelaide, a neighbouring observing station. The parabolic terms were, however, all insignificant and were not retained. In making allowance for the fact that no yields were available in 1923, the polynomial value of each rainfall distribution constant was calculated for that year and the difference, observed value—polynomial value, found in each case.

The quantities a' , b' , c' , and d' , which are proportional to the polynomial terms, were taken as independent variates in terms of which yields were to be expressed in the form of a multiple regression relationship. The standard technique is used to determine the partial regression coefficients, but before proceeding to the solution of the normal equations, the matrix of sums of squares and products of the independent variates must be corrected by making allowance for secular trend and omission of the year 1923.

With rainfall measured in the unit 10^{-3} in., the matrix of corrected sums of squares and products is

	a'	b'	c'	d'
a'	171396	-4398	-14289	-7934
b'	-4398	22179	-691	-282
c'	-14289	-691	12423	-5579
d'	-7934	-282	-5579	11858

and the inverse of this matrix is

7.618731	2.103194	14.189972	11.823744
2.103194	45.823220	7.720513	6.129333
14.189972	7.720513	128.808584	70.280276
11.823744	6.129333	70.280276	125.453847

each value being multiplied by 10^{-6} .

TABLE 7
CORRECTED SUMS OF PRODUCTS (YIELD AND RAINFALL DISTRIBUTION CONSTANTS)
Dimensions (bush.in.)

Plots	a'	b'	c'	d'
26-27	7.31530	-0.63527	-1.97764	0.71288
52-53	6.41082	-0.89363	-1.65307	0.45876
28-29	9.42050	-0.42372	-2.17046	0.75947
58-59	4.79836	-0.34972	-2.07570	1.16497
30-31	9.87960	-0.17377	-2.54519	1.07318
32-33	9.91132	0.00528	-2.78624	1.22241

After correction for secular trend and omission of the data for 1923, the sums of products of yield and the rainfall distribution constants are given in Table 7, yield being measured in bushels per acre and rainfall in inches, and with the same units the partial regressions of yield on the rainfall variates are as given in Table 8.

It is of interest at this stage to calculate how much of the annual variability in yield is expressible in terms of the quantity and distribution of seasonal rainfall. For example, with respect to plots 26-27 the sum of squares ascribable to the regression on all four variates is 502.61, but if a' had been the only measure of rainfall, the regression of yield on it would have been

$$7.31530/0.171396 = 42.68 \text{ bushels per acre per inch}$$

and the sum of squares due to this regression

$$7.31530^2/0.171396 = 312.22.$$

TABLE 8
PARTIAL REGRESSIONS OF YIELD ON RAINFALL DISTRIBUTION CONSTANTS
Dimensions (bush.in.⁻¹)

Plots	a'	b'	c'	d'
26-27	34.7635	-24.6234	-105.7361	33.0453
52-53	28.9300	-37.4166	-96.6184	11.6969
28-29	49.0620	-11.7053	-95.7936	51.5256
58-59	20.1420	-14.8184	-120.1057	54.8595
30-31	51.4774	-0.2562	-113.5696	71.5066
32-33	50.4396	7.0685	-132.2979	74.7594

The difference between these two sums of squares is the amount attributable to the regression on the distribution constants b' , c' , and d' . This series of calculations is summarized in Table 9 in an analysis of the annual variation of yield, plots 26-27.

TABLE 9
ANALYSIS OF VARIANCE (ANNUAL VARIATION IN YIELD, PLOTS 26-27)

Variation Due to	Deg. Freed.	Sum Sq.	Mean Sq.	Variance Ratio
Regression on a' , b' , c' , d'	4	502.61	125.65	14.87
Regression on a' alone	1	312.22	312.22	36.95
Difference (Regression on b' , c' , d')	3	190.39	63.46	7.51
Deviations from regression	19	160.55	8.45	
Total	23	663.16		

The regression on a' alone is highly significant and thus gives decisive information regarding the effect of quantity of rain, namely that the yield is increased on the average by

$$42.68/48 = 0.89 \text{ bushels per acre}$$

per inch of rain per six-day period.

The mean square for regression on b' , c' , and d' is also strongly significant, showing that the distribution of rainfall throughout the season has a marked effect on yield. The multiple correlation is $R = 0.87$ and the percentage of variance, A , ascribable to the average effect of the rain is 71 per cent.

Table 10 summarizes these analyses for the selected plots.

The regressions of yield on quantity of rain alone are

26-27	52-53	28-29	58-59	30-31	32-33
42.68	37.40	54.96	28.00	57.64	57.83

Comparison of these values with the partial regressions on a' given above in

TABLE 10
SUMMARY OF ANALYSES OF VARIANCE

Plot Number	Sums of Squares			Per Cent. Variance	
	Regression	Deviations from	Total		
	Formula (4 deg. freed.)	Regression (19 deg. freed.)	(23 deg. freed.)	R	(A)
26-27	502.61	160.55	663.16	0.87	71
52-53	383.99	239.66	623.65	0.78	53
28-29	714.20	286.24	1000.44	0.84	65
58-59	415.04	473.36	888.40	0.68	36
30-31	874.42	337.48	1211.90	0.85	66
32-33	959.96	284.66	1244.62	0.88	72
				Mean	61

Table 8. show that the latter are all smaller in magnitude and indicates roughly the degree to which the estimated average effect of the rainfall is modified when seasonal distribution is taken into account.

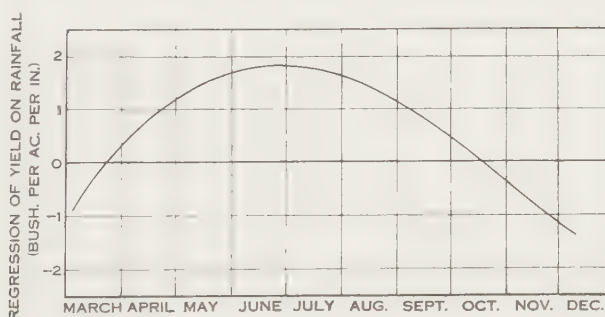


Fig. 9.—Regression function at Roseworthy (no manure treatment).

(v) *The Regression Functions.*—In order to construct regression functions for the various treatments, showing the estimated average effect of an additional inch of rain at any time in the season from March 1 to mid-December, the partial regressions of each set are multiplied by factors of the form

$$\frac{(2p)!}{(p!)^2 48.49 \dots (48 + p)},$$

where p takes successively the values 0, 1, 2, and 3. Substituting for p in this order the numerical values are $1/48$, $1/1176$, $1/19600$, and $1/299880$ and the four coefficients obtained from the partial regressions of plots 26-27. for

example, are 0.7242, -0.02094 , -0.005395 , and 0.0001102 . These coefficients are then multiplied in order by the polynomials tabulated by Fisher and Yates (1948) for the series $n = 48$, to give the sequence of points of the regression function. Figures 9, 10, and 11 show the course of this function throughout

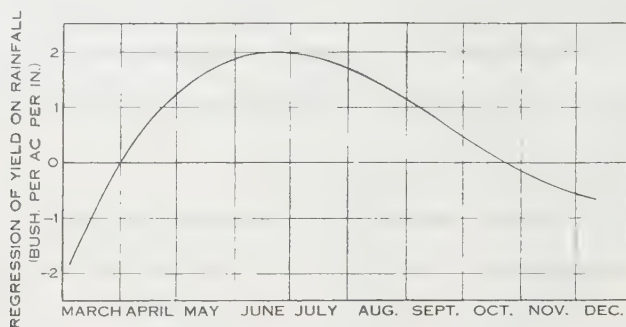


Fig. 10.—Regression function at Roseworthy (superphosphate 2 cwt. per acre).

the selected period. Only the average curves of the duplicates are shown in Figures 9 and 10, and in Figure 11 the curve is the average of two treatments since the individual functions are almost identical.

The three curves follow similar courses of a predominantly parabolic form but do differ in certain points. Similarity of form indicates the dominant role of seasonal conditions in contrast to the secondary influence that manurial treatment has in modifying the response of the crop, even in cases where the differences between the curves are significant. It must be remembered, however, that the sequence of years may be too short, in view of the extremely erratic nature of seasonal conditions, to establish the differences accurately.

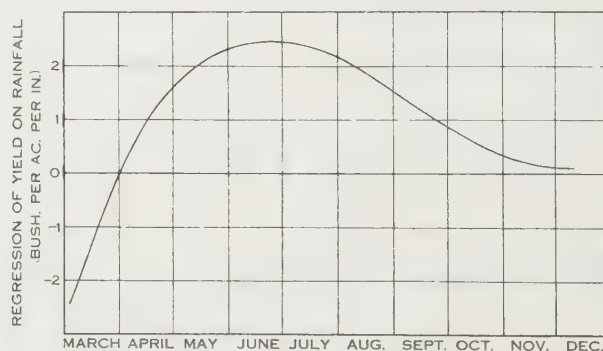


Fig. 11.—Regression function at Roseworthy (superphosphate 2 cwt. per acre and sodium nitrate 1 cwt. per acre).

The detrimental effect of excess rain in the initial stages, exhibited by all curves, is rapidly reduced, and by April 1 is transformed to a beneficial effect. The extent of the damage is greatest on plots 30-31 and 32-33, followed by

28-29 and 58-59, 26-27 and 52-53. From April the advantage to the crop increases under all treatments, reaching a maximum during the last 10 days of June; the greatest response is, as would be expected, on plots 30-31 and 32-33 (2.49), followed by 28-29 and 58-59 (1.98), 26-27 and 52-53 (1.78). Thereafter the response gradually diminishes and with the exception of plots 30-31 and 32-33 assumes negative values during the latter part of October and maintains them until mid-December. These observations are in the reverse order of those at the beginning of the season, but no special significance can be attached to the decreases noted in Figures 9 and 10 after the end of the second week in November, since from this date onward an increasing proportion of years occurs in which rains are too late to have any influence on the crop.

The presence of such pronounced decreases due to March rains was unexpected since on general grounds and from the evidence available it might be anticipated that the effects would be negligible. Several possible explanations of this observation immediately suggest themselves: (1) A spurious effect introduced through correlation of March rains with those of other parts of the season; (2) a chance correlation existing in the series of years under review; (3) rust epidemics associated with heavy rains of late summer (*vide* Cass Smith and Millington 1944); or (4) distortion at the ends of the range due to the inherent disadvantage of polynomial approximation; but none of them withstands close examination. The agreement of all plots and the magnitude of the initial negative regressions are, however, indicative of a genuine effect and this view is supported, though not with completely independent testimony, by Prescott's observation (1933) that, at Roseworthy, the correlation between yield of wheat and rains of the period January-March inclusive, immediately prior to seeding was -0.45 . The probability that such a result should be obtained by chance from uncorrelated material is approximately 1 in 50. Prescott examined data from three other South Australian stations, Minnipa, Booborowie, and Veitch, the correlations being respectively -0.22 , -0.23 , and -0.27 , and remarked that the series of four negative coefficients was suggestive of a harmful effect of these rains. Further consideration does, however, render untenable any suggestion that such a relation holds widely *under edaphic and climatic conditions similar to those of the stations mentioned*. In the first place, when the four coefficients are taken individually only that for Roseworthy is significant and, when taken conjointly, the probability of a chance result is about 1 in 10; secondly, data from stations similarly situated provide no evidence that the relation exists. It thus appears that some particular circumstance is associated with the locality. Since Roseworthy College is nearly centrally situated in the hundred of Mudlawirra its rainfall may be taken as representative of that area, and consequently if the feature observed is not confined to the College farm itself, it should appear on examination of the yields from the hundred. To test this point yields of the College and the hundred, for the same sequence of years 1905-30 inclusive, were correlated with the distribution constants a' , b' , c' , and d' and two regression functions were constructed. The first few polynomial values are:

Date		Roseworthy College Regression Coefficient (bush./ac./in.)	Hundred of Mudlawirra Regression Coefficient (bush./ac./in.)
March	3	-1.77	-0.26
	9	-1.39	0.01
	15	-1.04	0.25
	21	-0.72	0.46
	27	-0.43	0.66
April	2	-0.16	0.83
	9	0.09	0.97

thus establishing, beyond all doubt, the extremely localized character of the phenomenon.

The effect observed is attributable to a temporary depletion of the reserves of available plant nutrients, particularly nitrates, caused by a chain of circumstances depending ultimately upon the high degree of weed infestation, which, incidentally, was not confined merely to the experimental plots, but extended throughout the College area.

Early seasonal rains stimulate germination and rapid development of weeds which immediately draw on the nitrate supply generated in the fallows. Although this organic material is returned to the soil during preparations for seeding, its nitrogen would not become available again until decomposition occurred, and moreover, in the various stages of the latter process, in particular the initial stages, soil nitrates suffer a further reduction as micro-organisms draw on the supply to synthesize microbial protein. The influence on the regression function of nitrate deficiency caused in this way is transient since it is rapidly counterbalanced by opposing beneficial effects of later rains and nitrogen gradually becomes available. The crop is, however, deprived of a certain proportion of the nitrogen as some of the weed population survives to complete the life cycle. Two features of the analysis support this explanation of the negative March regressions. In the first place, the order of magnitude of the damaging effect of excessive March rains is the same as the order in decrement of yield which, as stated above, is itself due principally to infestation by weeds, and secondly, there is a response to nitrogen as indicated by contrasting the curve of Figure 11 with those of Figures 9 and 10. The general management of the farm was subjected to radical changes in 1932-33 so that further confirmation may be sought by examining data of the period 1933-48 inclusive. Only yields for the whole farm are available, and their correlation with March rainfall of the 16 years is -0.06, an insignificant value. The paucity of the data does not warrant any greater detail in the analysis.

One other point should be mentioned. It is known that heavy autumnal rains are very effective leaching agents (Prescott and Piper 1930). Penman (personal communication 1949) has pointed out that this is due to the structural condition of aggregated soil particles following the long arid summer season, and that a substantial proportion of nitrate leached to the subsoil may

disappear, probably by denitrification and subsequent dissipation of nitrogen in gaseous form. At Roseworthy, leaching, especially in the surface layers, may thus tend to accentuate the nitrate deficiency, but under the prevailing climatic conditions, it could not reasonably be regarded as a major contributor. In *all* the data herein examined, the only locality that shows definite evidence of nitrate deficiency, caused by leaching, and *sufficiently acute to give rise to negative regressions on rainfall*, is a small area of podsollic soils situated in southern Eyre Peninsula (*vide* Cornish loc. cit., Fig. 8).

(vi) *Significant Differences between Regression Functions.*—At this juncture it is perhaps worth while to give, at some length, a description of the significance test of the difference between two curves as a preliminary to contrasting the results obtained under the several treatments. Actually, the test can be made in two ways, depending on whether replicates of the treatments exist or not. If there is no replication, the test is made by comparing the variance ascribable to the rainfall curve fitted to the differences between the two treatments with deviations of the differences from the fitted curve. On the other hand, if replicates of the treatment exist, a rainfall curve is fitted to each replicate and the difference between the average curves of the treatments is then tested against the differences between rainfall curves of replicates of the same treatment. Obviously in this case, the first test can also be applied in comparing the average curves of the two treatments. The two tests are not necessarily equivalent, but the cogency of this statement may not be apparent until further details of each test, and the appropriate interpretation of the two results are given.

First test.—To illustrate this test consider the duplicate sets of yields from plots 26-27 and 52-53. In applying the test, a rainfall curve is fitted to the differences between the deviations of the observed yields in the two series from their respective linear trends in a manner similar to that used in finding the rainfall curves for individual plots. The same curve can obviously be obtained by taking, at all points, the difference between the curves already fitted to the two series of yields. This provides a very expeditious means for finding the actual curve but, in order to make the test of significance conveniently, the regression coefficients are required. It is not, however, necessary to start *ab initio*. Since the regression coefficients and product sums of yield with the rainfall distribution constants are available for each plot, it can be shown that the quantities required in making the significance test can be obtained by taking differences. Thus from Tables 7 and 8, the product sums and regression coefficients, together with their differences, are as follows:

Products of Yield with	Plots		Difference
	26-27	52-53	
a'	7.31530	6.41082	0.90448
b'	-0.63527	-0.89363	0.25836
c'	-1.97764	-1.65307	-0.32457
d'	0.71288	0.45876	0.25412

Regression on

a'	34.7635	28.9300	5.8335
b'	-24.6234	-37.4166	12.7932
c'	-105.7361	-96.6184	-9.1177
d'	33.0453	11.6969	21.3484

and the sum of the squares ascribable to the regression of the differences between yield on the rainfall distribution constants is thus

$$5.8335 \times 0.90448 + \dots + 21.3484 \times 0.25412 = 16.97.$$

The total sum of squares of the differences is now required. If y stands for an observed yield, Y for the predicted yield according to the linear trend, and subscripts 1 and 2 are added to distinguish the replicates, this sum is

$$S\{(y_1 - Y_1) - (y_2 - Y_2)\}^2 = S(y_1 - Y_1)^2 + S(y_2 - Y_2)^2 - 2S(y_1 - Y_1)(y_2 - Y_2).$$

The two sums of squares on the right-hand side are already available and their actual values are, from Table 10, 663.16 and 623.65 respectively. The sum of products is obtained in a similar manner to those given in Table 7, making due allowance for secular trend and omission of the year 1923. The corrected sum is 536.32 and consequently the sum of the squares of differences is 214.17. This series of operations can then be summarized in the analysis of variance of Table 11. Obviously, no significant difference exists between the two curves on the basis of this test.

TABLE 11

ANALYSIS OF VARIANCE (DIFFERENCE BETWEEN RAINFALL CURVES, PLOTS 26-27 AND 52-53)

Variation due to	Deg. Freed.	Sum Sq.	Mean Sq.
Regression of difference between replicate curves on a' , b' , c' , d'	4	16.97	4.24
Regression on a' alone	1	4.77	4.77
Difference (regression on b' , c' , d')	3	12.20	4.06
Deviations from regression	19	197.20	10.38
Total	23	214.17	

A similar test of the difference between the rainfall curves of plots 28-29 and 58-59 showed that the regression on a' alone was greater in the former replicate; the difference is significant at the 5 per cent. point but in other respects the curves may be regarded as equivalent. Re-examination of the original data did not disclose any peculiar circumstance relating to plots 58-59 which might reasonably account for the discrepancy between the regressions.

Second test.—Since there is no evidence to show that the autumnal and spring dressings of sodium nitrate differ in their effects, the series 30-31 and 32-33 were taken as replicates and the second test may be illustrated by comparing their average curve with the average of the duplicates 26-27 and 52-53. The appropriate product sums and regression coefficients from Tables 7 and 8

respectively are first averaged and then differences between the averages are taken. The figures are as follows:

Product of Yield with	Average of Plots		Difference
	30-31 and 32-33	26-27 and 52-53	
a'	9.89546	6.86306	3.03240
b'	-0.08425	-0.76445	0.68020
c'	-2.66571	-1.81536	-0.85035
d'	1.14779	0.58582	0.56197
Regression on			
a'	50.9585	31.8468	19.1117
b'	3.4062	-31.0200	34.4262
c'	-122.9338	-101.1772	-21.7566
d'	73.1330	22.3711	50.7619

The sum of squares ascribable to the regression of the difference on the rainfall distribution values is thus 128.40.

The total sum of squares of the differences between the average deviations is now required. If, as before, y and Y stand for observed and predicted yields respectively, subscripts 1 and 2 distinguish the replicates and superscript dashes are added to denote manurial treatment, the sum of squares (superphosphate and sodium nitrate - no manure) is

$$\begin{aligned}
 & S\left\{\frac{1}{2}[(y'_1 - Y'_1) - (y'_2 - Y'_2)] - \frac{1}{2}[(y''_1 - Y''_1) + (y''_2 - Y''_2)]\right\}^2 \\
 &= \frac{1}{4}\{S(y'_1 - Y'_1)^2 + S(y'_2 - Y'_2)^2 + S(y''_1 - Y''_1)^2 + S(y''_2 - Y''_2)^2 \\
 &+ 2[S(y'_1 - Y'_1)(y'_2 - Y'_2) + S(y''_1 - Y''_1)(y''_2 - Y''_2)] \\
 &- 2[S(y'_1 - Y'_1)(y''_1 - Y''_1) + S(y'_1 - Y'_1)(y''_2 - Y''_2) \\
 &+ S(y'_2 - Y'_2)(y''_1 - Y''_1) + S(y'_2 - Y'_2)(y''_2 - Y''_2)]\}.
 \end{aligned}$$

Only the last four product sums need be calculated since the remaining quantities are available from previous tests. Numerically the sum of squares is 280.47 and the analysis of variance is given in Table 12.

In conducting the second test, a comparison is made between the mean square 32.10 with 4 degrees of freedom and the mean square 2.91 with 8 degrees of freedom; in this illustration the difference is strongly significant, $0.001 < P < 0.01$.

The analysis may be carried further by partitioning the sum 128.40 into two of its components

- (i) regression on a'
- (ii) regression on b' , c' , and d' ,

and comparing the mean square of each with its corresponding components in the sum 23.25 with 8 degrees of freedom. This test shows that the regressions on quantity of rain and its distribution are individually significant, $0.01 < P < 0.05$, in each case, and consequently there is a real difference between the average curves of plots 30-31, 32-33 and 26-27, 52-53.

After comparing the two tests, it is apparent that only in the second is account taken of soil heterogeneity. Consequently, any significant difference

demonstrated by the first test is not necessarily attributable to differential treatment as it may be due to inherent differences between the plots. Apart from this, another important difference exists between the two tests. Suppose the treatments to be compared are replicated and the average regression of each set of replicates is taken. Consider one of the treatments. If the sequence of years under examination can be regarded as a random sample of rainfall values, and is of sufficient length to sample adequately the range of rainfall variation, the expectation is that the average regression, obtained from any other sample containing the same number of seasons, will only differ from that calculated on the first sample by more than twice the standard deviation of the first regression (as computed from the deviations from the average regression line) in 5 per cent. of trials. The two regressions may or may not be

TABLE 12

ANALYSIS OF VARIANCE (DIFFERENCE BETWEEN AVERAGE CURVES, PLOTS 30-31 AND 32-33
LESS PLOTS 26-27 AND 52-53)

Variation due to	Deg. Freed.	Sum Sq.	Mean Sq.	Variance Ratio
Total	23	280.47		
Regression of difference between average curves on a' , b' , c' , d'	4	128.40	32.10	11.03
Deviations from regression	19	152.07	8.00	
Regression of difference between replicate curves on a' , b' , c' , d'				
(i) Plots 26-27 and 52-53	4	16.97	23.25	2.91
(ii) Plots 30-31 and 32-33	4 } 8	6.28		
Deviations from regression				
(i) Plots 26-27 and 52-53	19	197.20	234.23	6.16
(ii) Plots 30-31 and 32-33	19 } 38	37.03		

really different, but the important point is that their difference is unlikely to be significant using sampling errors calculated by the first test. As replicates exist, a separate regression can be fitted to each and the significance of the average regression may be tested by comparison with the variation among regressions of individual replicates. Since the experience of all replicates is based upon an identical sequence of seasons, it may be anticipated that the random errors associated with the replicates are on a smaller scale, and if the effects due to soil heterogeneity (between replicates) are small or non-existent, the average regression calculated from the first sample of seasons would probably be significant. The same remark applies equally well to the average regression based on the second set of seasons. Thus the average regressions may be established as individually significant, but it is also possible that their difference is significant, or that they exhibit opposing effects due to rainfall, especially if the two samples of seasons differ markedly. The smaller error in the second test cannot thus be relied upon to provide an accurate guide as to

the nature of the regression obtained from a different sample of seasons, and consequently conclusions based on the second test are valid only for the particular set of seasons which have actually been experienced. If soil heterogeneity makes no contribution to the difference between the regressions, the first test is the more exacting when the tests are not equivalent because it accounts for all sources of variation of which account is taken in the second test apart from soil heterogeneity. This point is well illustrated in the example used above in the second test of significance. Soil heterogeneity does not affect the duplicates and, as shown, the difference between the average regressions is strongly significant ($0.001 < P < 0.01$) on the second test. On the other hand, comparison of the mean square 32.10 with the mean square 8.00 (19 degrees of freedom) by the first test, gives a variance ratio of 4.01 for which $0.01 < P < 0.05$.

Comparative tests among the three treatments gave the following results:

Superphosphate plus sodium nitrate	v.	No manure	Significant differences between the regressions on both quantity and seasonal distribution of the rains.
Superphosphate plus sodium nitrate	v.	Superphosphate	Significant difference between regressions on quantity of rain only.
Superphosphate	v.	No manure	No significant differences.

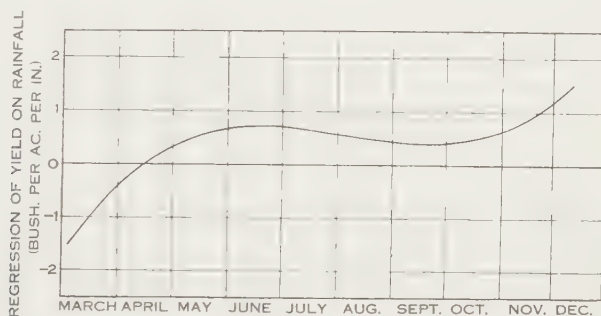


Fig. 12.—Difference between regression functions of Figures 9 and 11.

The first of these comparisons provides the greatest contrast and the regression function representing it is illustrated in Figure 12. The two salient features are the heavier damage due to excess March rain and the greater response to winter rains on plots 30-31 and 32-33. The former has been covered in the discussion above, while the latter follows from the response of the crop to nitrogen and phosphorus supplied in the fertilizer. The treatment replaces some of the nitrogen rendered unavailable and may even assist in the liberation of a certain proportion of it by facilitating decomposition of the organic

material ploughed into the soil. The reaction must occur in this way since on well-prepared fallows nitrification generates sufficient nitrate to meet the demands of cropping so that normally there is no response to dressings of nitrogenous fertilizers.

The significant difference on quantity of rain in the second comparison follows also from the stimulus given by the additional nitrate.

The negative result of the third comparison was unexpected since it was anticipated that a difference in favour of superphosphate would be obtained in accordance with the established observations that soils of the wheat belt are deficient in available phosphorus and that phosphatic fertilizer induces early development of the wheat plant. The response of the crop to seasonal rains must necessarily be intimately related to the fertility of the soil, but the dominating effect of rainfall, coupled with the fact that the sample of years is too small to allow the disturbing influence of erratic seasons to be smoothed out, make it impossible to establish any but major differences. The solution of the main problem is complicated also by the competitive effect of the weeds since the shortage of nitrogen on the plots treated with phosphate alone no doubt influences the response to phosphorus. This factor is probably not operative on the plots treated with both superphosphate and sodium nitrate after application of the latter.

(b) Minnipa

The yield data were taken from records of the State experimental station at Minnipa for the period 1916-44 inclusive. Farming commenced in 1916 on virginal sandy mallee soils of granitic origin, which in their original state carried mallee eucalypts (*E. gracilis*, *E. dumosa*, and *E. oleosa*). The property was operated as an experimental station until 1930 and subsequently on a basis of share-farming. All wheat crops have been grown with superphosphate on mixed areas of fallow, stubble, and new land, the total area in any

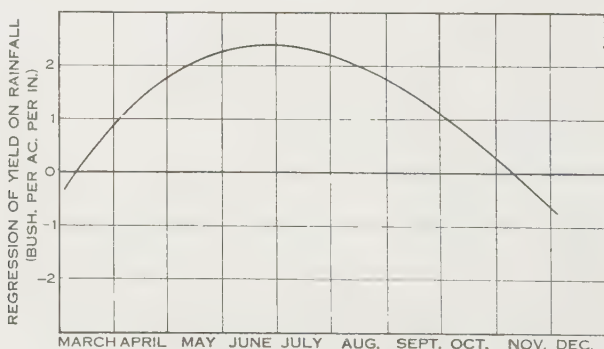


Fig. 13.—Regression function at Minnipa.

year ranging from 100 to 1,000 acres. Rainfall data were recorded in the township of Minnipa adjacent to the farm. The method of analysis was similar to that used for Roseworthy, seasonal weather being represented by rains of the period March 1-December 8 to which a polynomial of the third degree was fitted. The regression function is illustrated in Figure 13.

(c) *Kimba*

This record was obtained from a large private property on which approximately 600 acres of wheat were harvested annually, and extends over the period 1917-43 inclusive. The farm was established in 1916-17 on virginal sandy mallee which in its native state carried *Callitris* spp. in addition to typical mallee. All crops have been supplied with superphosphate and in the early years were sown on fallow, stubble, and new land, but latterly in 4 course rotations. Rainfall observations were taken in Kimba nearby. Seasonal rains were computed for the period March 1-December 8 and the analysis was similar to that used for Roseworthy. The regression function is given in Figure 14.

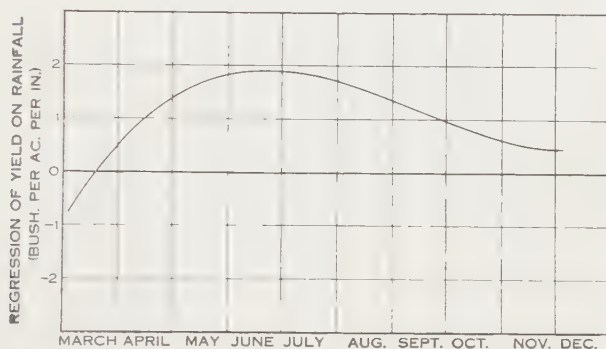


Fig. 14.—Regression function at Kimba.

(d) *Veitch*

The yield data were recorded at the State experimental farm, Veitch, for the years 1909-30 inclusive. Farming was commenced in 1909 on virginal sandy mallee soils which carried mallee eucalypts with only a sparse bushy undergrowth indicative of the low rainfall, and all wheat crops have been grown with superphosphate on mixed areas of fallow, stubble, and new land, the total area in any year ranging from 100 to nearly 1,000 acres. Rainfall observations were taken at Veitch's Well on a site adjoining the farm. As the data were recorded only by calendar months, seasonal conditions could not be considered in detail and recourse was therefore taken to an analysis by multiple regression in which rains of the periods April-May, June-July-August, and September-October were taken as variates. The partial regressions in bushels per acre per inch are

April-May	1.89 ± 0.486	} 18 degrees of freedom
June-July-August	2.11 ± 0.264	
September-October	1.54 ± 0.354	

(e) *Booborowie*

This State experimental farm formed part of North Booborowie station which for many years had carried sheep and was repurchased by the State for closer settlement in 1911. The yields were obtained from areas ranging from

100 to 400 acres during the period 1912-30 inclusive. The soil is a typical red brown earth which in its native state carried bluegum (*Eucalyptus leucoxylon*) on the flats and she-oak (*Casuarina stricta*) on the rises with a dense covering of *Lomandra*, Wallaby grass (*Danthonia* spp.), and Spear grass (*Stipa* spp.). All wheat crops were grown with superphosphate on fallow. Rainfall observations were taken on the property and as the data were available only in calendar months, the analysis was similar to that used for Veitch. The partial regressions in bushels per acre per inch are

April-May	1.10 ± 0.375	} 15 degrees of freedom
June-July-August	1.80 ± 0.255	
September-October	0.78 ± 0.330	

(f) Saddleworth

This record of yield data, which extends over the period 1897-1943, was taken from a large private holding near Saddleworth. The property is comprised almost equally of two soil types, a black earth (chernozem) and a type intermediate in grade between a red brown earth and a podsol. Wheat has been sown with superphosphate on areas averaging 500 acres annually in various crop rotations involving 3-7 courses. Rainfall observations were taken in the nearby township of Saddleworth and the season has been represented by rains of the period March 1-December 31, the analysis being similar to that used for Roseworthy except that each season's data were fitted with a polynomial of the 5th degree. The regression function is given in Figure 15.

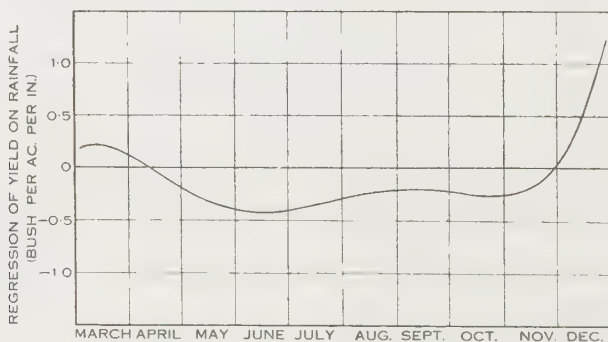


Fig. 15.—Regression function at Saddleworth.

(g) Lameroo

The private property from which this record for the years 1911-46 inclusive was taken, is situated near Lameroo and was established in 1910-11 on virginal transitional mallee-solonetz soil carrying in its native state mallee (*E. incrassata*) and broombush (*Melaleuca uncinata*). At this particular site the soils tend toward complete solonization. Wheat crops have all been supplied with superphosphate and have been sown on mixed areas, ranging from 100 to 450 acres, of fallow and stubble and latterly in rotations. Rainfall observations were taken on the property. The season was represented by rains of

the period March 1-December 20, the analysis being similar to that used for Roseworthy. The regression function is illustrated in Figure 16.

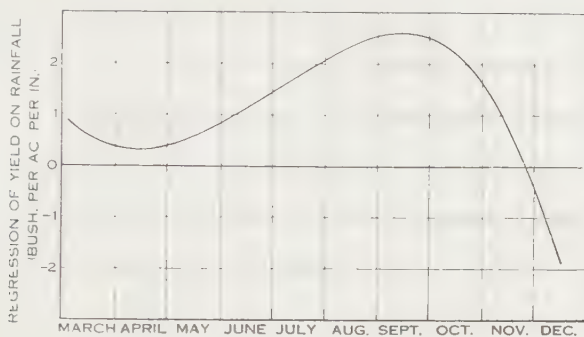


Fig. 16.—Regression function at Lameroo.

(h) General Remarks

In the analyses of the data listed in Sections III(b) to III(g) above, secular variations in yield and/or rainfall, wherever they occurred, have been eliminated before correlating the variables.

Considering the soils of the wheat belt broadly, the more lightly textured mallee types may be represented by Minnipa, Kimba, and Veitch, the heavier loamy mallee types by Roseworthy, transitional mallee-solonetz by Lameroo, red brown earths in zones of less than 15 in. seasonal rainfall by Booborowie, and red brown earths with their associated soils in zones of more than 15 in. seasonal rainfall by Saddleworth (*vide* Cornish loc. cit., Fig. 8).

The results obtained from these individual records substantiate the observations and general conclusions given in Section II(c), and vindicate the argument used in the choice of the rainfall variates and analytical technique. Regressions on both quantity and seasonal distribution of rainfall are significant at Kimba, Minnipa, and Lameroo, but not at Saddleworth. After taking the average curve of the three treatments at Roseworthy to represent that locality, comparative tests among the regression functions yielded significant differences in all cases except the contrast between Minnipa and Kimba. It is, however, certain that, if the particular circumstances noted at Roseworthy had not applied, the regression function would not have differed significantly from either Minnipa or Kimba. The partial regressions obtained at Booborowie indicate that the regression function of the red brown earths also assumes a parabolic form.

Average values of the ordinates for the periods April-May, June-July-August, September-October, taken from the regression functions of Figures 9 to 11, 13, 14, and 16, together with means of the components of seasonal rainfall, are set out in Table 13. These regressions, and the corresponding quantities from Veitch and Booborowie, are in close agreement with the coefficients obtained for hundreds on the same broad soil types in which the standard of

wheat farming attains a reasonable level. Average figures for the hundreds are naturally somewhat lower since they have been derived from yields obtained under general industrial conditions and in certain areas are influenced also by the fact that seasonal rains approach their optimal values.

TABLE 13
AVERAGE REGRESSIONS AND COMPONENTS OF SEASONAL RAINFALL FROM
SELECTED STATIONS

Station	Regressions (bush./ac./in.)			R	Components of Seasonal Rainfall (in.)			Total
	Apr.- May	June- July- Aug.	Sept.- Oct.		Apr.- May	June- July- Aug.	Sept.- Oct.	
Roseworthy Fig. 9	1.1	1.6	0.5					
Fig. 10	1.1	1.8	0.6					
Fig. 11	1.4	2.2	1.0					
Roseworthy mean	1.2	1.9	0.7		3.11	6.30	3.85	13.26
Minnipa	1.7	2.2	1.2	0.85	2.03	5.66	2.41	10.10
Kimba	1.3	1.8	1.0	0.79	2.00	5.10	2.65	9.75
Mean	1.5	2.0	1.1					
Veitch	1.9	2.1	1.5	0.93	1.85	3.60	2.63	8.08
Booborowie	1.1	1.8	0.8	0.91	2.78	6.81	4.00	13.59
Saddleworth	—	—	—	—	3.80	7.25	3.93	14.98
Lameroo	0.5	1.7	2.4	0.78	2.81	5.28	3.36	11.45

The curve for Lameroo contrasts strongly with the remainder, and confirms the observation made above that transitional mallee-solonetz soils behave differently from other soil types within the same zone of rainfall, but the cause of this is at present obscure. At Saddleworth, average rainfall is very nearly optimal at all stages of the season. Since harvesting normally takes place in late December, the rains of November should be included in the seasonal total; this gives an amount of 16.15 in. which would point to the fact that the estimate of 17 in. for red brown earths and associated types given in Section II(c) is too high, even after allowing for errors of estimation. This view may be tested by examination of the records from the Waite Agricultural Research Institute. The soil at this station is a typical red brown earth and the mean values of the components of seasonal rainfall are April-May 5.03 in., June-July-August 9.14 in., September-October 4.61 in., and November 1.47 in. It is known (Cornish unpublished data) that April-May rains are considerably in excess of the optimum; June-July-August rains are moderately so; while September-October and November rains are close to their optimal values in this locality. As at Saddleworth, November rains must be included in the seasonal total, and consequently, in districts where harvesting normally occurs during the latter half of December, it appears that the estimate of 8.8 in. for winter rains is between 1 and 2 in. too high, but that the remaining two estimates are fairly accurate. On the other hand, in regions where the crop reaches

maturity between mid-November and mid-December, it is probable that the figure given is substantially correct. The estimated optimal seasonal total for the heavier mallee soils stands in proper relation to that of the red brown earths since effective rainfall is lower in the climatic zone of the former soil types.

The values of the quantity *A* are Minnipa 67 per cent., Kimba 54 per cent., Veitch 83 per cent., Booborowie 79 per cent., and Lameroo 55 per cent., which in combination with the figures from Roseworthy, show that approximately 65 per cent. of the annual variance of yield is ascribable to the average effects of the rainfall, thus strongly supporting the contention made above that rainfall is a sufficiently reliable index of seasonal weather in the wheat belt of South Australia. The result also indicates that in the near future it should be possible to bring fairly accurate forecasts within reach of the State and the industrial farmer when the effects of rainfall have been more accurately assessed by taking account of the information available in additional data.

IV. COMPARISON WITH PREVIOUS WORK

Perkins and Spafford (*loc. cit.*) in two early reports on the work of the permanent experiment field at Roseworthy summarized their conclusions after studying the effects on yield of seasons favourable and unfavourable to wheat. Their examination was confined to the data accumulated during the first 10 years of the experimental work, but in framing their reports, they no doubt drew upon experience gained from cropping in the district over a longer term. The summary of their conclusions from the second report is as follows:

"In the Roseworthy district, and those similarly situated, 15 in. to 16 in. of rain well distributed over the growing period of cereals—April to November—render possible yields of 28 bush. to 30 bush. of wheat or 3 to 3½ tons of hay.

"In the same districts the most favourable rain distribution for maximum crops is represented by good early seeding rains aggregating 4 in. to 5 in. in April and May, followed by 4 in. to 5 in. in June and July, and by 6 in. to 7 in. between August 1 and October 31. November rains are, as a rule, without significance in seasons that are otherwise favourable.

"In these districts the seasonal factors exercising most powerfully a depressing influence on yields, whether of grain or of hay, are as follows, in order of relative importance: (*a*) Unduly late seeding rains; (*b*) a shortage of seeding rains followed by a dry winter; (*c*) a shortage of spring rains following on an unfavourable winter; (*d*) unseasonably sudden hot weather accompanied by northerly winds in October or even in November; and (*e*) moist warm weather in October and November leading to attacks of red rust." In both reports considerable emphasis was laid upon the importance of seeding and winter rains. The conclusions arrived at here, using exact statistical methods and much more data reaffirm their findings with greater precision, and in fact extend them to a major proportion of the wheat belt.

In a general survey of the relation between yield and seasonal rainfall for the principal districts of South Australia, Perkins (*loc. cit.*) discussed,

inter alia, the relative effectiveness of April-November rains and summarized the position thus:

"In each district for every additional inch above April-November rainfall requisite to economic crops, given satisfactory distribution, we may anticipate increases in yield above the minimum of

1½ bushels in the Upper North
1 bushel in the Lower North
½ bushel in the Central District."

The districts to which Perkins referred are three of the former statistical subdivisions of the State. Upper North corresponds almost identically with Flinders (*vide* Fig. 1); Lower North to Goyder, northern Yorke and hundreds on the northern boundary of Light; and Central is comprised mainly of the remaining parts of Yorke and Light. The responses per inch of rainfall may then be compared with the regression coefficients plotted in Figures 2 to 6. Thus Perkins's three estimates, in the order given above, correspond approximately to the average values of the following groups:

1. Desert loams of group (*a*) in the three parts of Figure 6.
2. Red brown earths that have $x_1 < 2\frac{1}{4}$ in., $x_2 < 5\frac{1}{2}$ in., and $x_3 < 2\frac{1}{4}$ in. in Figure 2.
1. Red brown earths for which $2\frac{1}{4}$ in. $< x_1 < 3$ in., $5\frac{1}{2}$ in. $< x_2 < 6\frac{1}{2}$ in., and $2\frac{1}{4}$ in. $< x_3 < 3\frac{1}{2}$ in. in Figure 2.
2. Loamy mallee soils of group (*b*) that have $b_1 > 0.9$, $b_2 > 1.5$, and $b_3 > 0.7$ in Figure 3.
1. Red brown earths that have $x_1 > 3$ in., $x_2 > 6\frac{1}{2}$ in., and $x_3 > 3\frac{1}{2}$ in. in Figure 2.
2. Loamy mallee soils of group (*b*) that have $b_1 < 0.9$, $b_2 < 1.5$, and $b_3 < 0.7$ in Figure 3.

Perkins also gave a table showing the ideal distribution of so-called "useful" rain corresponding to requirements of the bulk of South Australian wheat-growing areas. The figures given are substantially the same as those quoted from his previous paper (Perkins and Spafford *loc. cit.*).

In reporting the results of an extensive research on the water requirements of farm crops under Victorian conditions, Richardson (*loc. cit.*) gave transpiration ratios of 395 for dry matter and 1067 for grain as averages of winter-grown wheat. Taking these figures as representing an average of seasons, he computed that, for moderate rainfall, each inch of rain transpired by the crop is capable of producing approximately $3\frac{1}{2}$ bushels per acre and then compared actual production with this hypothetical figure. For both Victoria and South Australia, seasonal rainfall was taken as that occurring during the period April-October inclusive and, in order to provide an average, representative of the main areas in each State, the mean of a number of selected stations was taken. In both States a very close relationship between State yield and average composite seasonal rainfall was found. The period under review embraced the years 1892-1921 inclusive and on partitioning it into three decennial periods, the following ratios were found:

Period	Average Yield (bush./ac.)		Composite Seasonal Rainfall (in.)		Bush./ac./in. of Seasonal Rain	
	Vict.	S. Aust.	Vict.	S. Aust.	Vict.	S. Aust.
1892-1901	7.65	4.73	11.49	11.55	0.67	0.41
1902-1911	10.50	9.62	11.63	12.18	0.90	0.79
1912-1921	12.62	10.47	11.59	11.18	1.09	0.94
Mean	10.26	8.27	11.59	11.64	0.89	0.71

Since the average rainfall in the several periods remains practically constant, the increases in the ratios show the extent to which wheat farming had progressed toward more effective utilization of soil moisture. Richardson observed that the transpiration rate increases exponentially during the early stages of growth and reaches a maximum during October which, with winter-grown cereals, follows closely after flowering, and usually coincides with stringent atmospheric conditions. The fact that approximately 40 per cent. of the total water used by the crop is transpired during October suggested that spring rains prior to, and during, that month should exercise a pronounced effect on the ultimate yield.

Following on Richardson's work, Barkley (*loc. cit.*) investigated the relationship between yield and monthly rainfall for the Wimmera district of Victoria, and found certain strong correlations. Thus he states:

"The outstanding feature of the rainfall effect in Australia is the definite limits of the critical seasons. In Victoria, for instance, eighty per cent. of the variations in the wheat harvest can be ascribed to the rainfall of August and September, leaving only twenty per cent. of the departures from an average yield to be accounted for by (*a*) moisture stored in the fallow, (*b*) May to July rains, and (*c*) the October rainfall. Of these subsidiary influences outside the critical spring months the falls in June are most important, and provide the earliest clue to the opening of the season."

He noted that the response in yield to increasing rainfall was logarithmic, the spring rains of August and September adding to the yields in the proportions of 8.22, 3.65, 2.03, 1.55, 1.01, and 0.98 bushels per acre for each successive inch up to the sixth. As already stated no such direct evidence of an approach to the optimum could be found in the data herein examined.

Barkley laid great stress upon the existence of these so-called critical periods and nominated them for other parts of the Australian wheat belt. For example, June was given as the critical month on the west coast of South Australia (Nuyts and Eyre of Fig. 1) and the Darling Downs (Queensland), and August in the Lower North of South Australia. If Barkley's statements are accepted the rains of June alone, and August alone would be expected to exercise strong control over yields in the respective districts mentioned, but it is clear that this is not the case. Whatever may be expected on physiological grounds on the basis of such work as that of Richardson, the results of the

present investigation show that, in the environment to which they are applicable, the influence of rainfall on yield is strongly marked throughout practically the entire growing season and is not confined to a few relatively short intervals of time.

V. ACKNOWLEDGMENTS

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THE ENUMERATION OF HEATED BACTERIAL SPORES

I. EXPERIMENTS WITH *CLOSTRIDIUM BOTULINUM* AND OTHER SPECIES OF *CLOSTRIDIUM*

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Summary

Experiments with 13 strains of types A and B *Cl. botulinum* have shown that the detection and enumeration of spores surviving lethal heating become increasingly difficult as the period of heating is increased. Media which are very satisfactory for enumerating unheated spores may be unsatisfactory for enumerating spores which survive the heat treatment. By comparison with unheated spores the numerical estimates of spores surviving severe heat treatments were more dependent on thioglycollate in the medium and on the pH and temperature during incubation. As the amount of heating was increased, the surviving spores became progressively more sensitive to inhibitors in the medium. These inhibitory substances were present in all media used, and their effects were counteracted under a wide range of conditions by the incorporation of approximately 0.1 per cent. starch in the medium. The starch supplement produced no significant effect on the estimates of unheated spores. The effects of starch are a feature of the amylose portion of the undegraded starch molecule which, like charcoal or serum albumin, acts by adsorbing inhibitors from the medium. The probability that at least part of the inhibition is due to unsaturated fatty acids is discussed.

The heated spores of several other species of *Clostridium* show a similar response to the inclusion of starch in the medium.

I. INTRODUCTION

In attempting to obtain more precise information on some of the factors affecting the thermal resistance of bacterial spores, particular attention was paid to the use of solid agar media for the enumeration of the spores remaining viable after various periods of heating. Some of the preliminary experiments were carried out with Australian strains of *Cl. botulinum*, information being desired on the thermal resistance of its spores. It was then discovered that various types of medium frequently gave divergent estimates of the numbers of spores surviving heat treatment even though each medium gave a similar estimate of the viable unheated spores, a phenomenon which had been described previously in experiments on heat resistance (Curran and Evans 1937). In the course of attempts to devise more reliable methods for the enumeration of spores surviving heat treatment it was found that the addition of small amounts of starch was of considerable value. A preliminary note of these findings has been published (Olsen and Scott 1946), and it is

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the purpose of the present paper to record the conditions under which starch was found effective and to show that, like charcoal or serum albumin, it acts by adsorbing inhibitory substances from the medium in which the spores germinate.

II. GENERAL METHODS

(a) *Spore Suspensions*

Spore suspensions were prepared by harvesting from various media, usually liquid, and washing twice in M/15 phosphate buffer of pH 7.0. Suspensions were examined microscopically and, if necessary, treated to ensure freedom from clumps. They were then heated to destroy vegetative cells by immersing sealed tubes for 10 minutes in a water-bath at 80°C. Concentrated suspensions so prepared usually contained between 10^6 and 10^8 viable spores per ml., and from them more dilute suspensions containing about 10^4 to 10^5 per ml. were prepared by dilution in the same buffer. All suspensions were stored, with precautions against evaporation, at 0. to 1°C.

(b) *Conditions of Heating*

Heating was carried out in thermostatically controlled ($\pm 0.3^\circ\text{C}.$) stirred paraffin oil baths operating at 100, 105, 110, or 115°C. Suspensions for heating were sealed in hard glass tubes which were totally immersed in the baths. The times of immersions have been corrected for the thermal lag as determined experimentally. Except where otherwise specified, all suspensions were heated in M/15 phosphate at pH 7.0. In these experiments very precise control of temperature was not attempted as the principal aim was not the exact evaluation of rates of destruction under specified conditions, but rather to establish the validity of cultural procedures for enumerating the true number of survivors. In the experimental results presented below, all comparisons of nutritive and cultural conditions have been made with spores exposed to identical heat treatments; in any one experiment the various media were inoculated with aliquots from a single tube or, where necessary, from the pooled contents of several tubes.

(c) *Viable Counts*

The initial numbers of viable spores were determined by estimation of the numbers producing visible colonies in solid agar media incubated under the conditions specified. It was found to be convenient to use the oval tubes described by Miller, Garret, and Prickett (1939). Each tube contained 20 ml. of agar and, unless otherwise specified, was incubated in air. No correction was made for the surface layer of approximately 10 per cent. of the medium in which *Clostridium* colonies failed to develop, but the constancy of this error was ensured by a simple method of distributing the inoculum uniformly with the aid of convection currents. In many experiments the progress of disinfection was followed by viable counts made after various periods

of heating. In other experiments, however, the performance of the media was tested simply for estimating unheated and heated spores, and in such cases estimates were made of the initial number of unheated spores and again after such a period of heating as was required to reduce the viable number to less than about one per cent. of the initial number. The general plan has been to inoculate duplicate tubes using decimal dilutions of the inoculum. In these circumstances, therefore, greatest emphasis has been placed on the general reproducibility of results rather than on obtaining rigorous statistical proof of differences within any one experiment.

(d) Media

Special care was taken to ensure that all media were prepared according to the formulation and procedures required. Records were maintained of the actual quantities of materials used in preparing each lot of medium and at the time of preparation each batch was identified with a serial number. In any one experiment modifications or supplements to a basal medium were always made with aliquots of the same batch. Unless otherwise indicated, supplements were added before the final autoclaving.

As a precaution against contamination by certain metals, all media were prepared with glass-distilled water, and infusions were made according to the procedures of Wright (1933). Brain heart infusion agar was prepared by adding 1.0 per cent. agar to the recipe for brain heart infusion given in the Difco Manual (1944). Pork infusion agar was prepared according to the formula of Brewer (1940), the methylene blue and sodium thioglycollate being added just prior to autoclaving. Sodium thioglycollate was added at 0.01 moles per litre by neutralizing a measured quantity of normal soda with pure thioglycollic acid just prior to use. Other meat infusion media were prepared by substituting the appropriate animal tissue for the pork used in this medium. Peptic digest medium and cooked meat medium were prepared as described by Dubovsky and Meyer (1922). Difco brand peptones were used throughout and the usual solidifying agent was 1.0 per cent. of air-dried agar (Davis Gelatine (N.Z.) Ltd.), media made with this agar being comparable in gel strength with 1.5 per cent. of Japanese or American (Difco) agar.

(e) Cultures

The following strains of *Cl. botulinum* were of Australian origin; Q6, Q7, L4, and S3 (type A), and 968, 1787, 3149, A12, and L12 (type B). The American strains of this organism which were tested, 62A, 109A (type A), and 112B, 213B (type B), were obtained from Professor K. F. Meyer. Cultures of *Cl. histolyticum*, *Cl. oedematiens*, *Cl. bifermentans*, *Cl. tetani*, and *Cl. botulinum* (type C) were obtained from Professor H. R. Carne.

Two organisms commonly used in heat resistance studies, *Clostridium* No. 3679 and *Cl. thermosaccharolyticum* No. 3814, were obtained from Dr. E. J. Cameron, of the N.C.A. Research Laboratories, Washington, U.S.A.

III. RESULTS OF EXPERIMENTS WITH *CLOSTRIDIUM BOTULINUM*(a) *The Performance of Media in Relation to the Amount of Heating of the Spores*

In some preliminary estimates of the heat resistance of the spores of some Australian strains of *Cl. botulinum* the living spores were enumerated in deep tubes of brain heart infusion agar. This medium, which had been found to be of considerable utility for the cultivation of various aerobic and anaerobic bacteria was, however, less dependable than liquid cooked meat medium for the detection of spores surviving heat treatment. Efforts were made to improve the performance of solid agar media to an extent which would permit reliable evaluation of rates of death, in order to avoid using liquid cooked meat medium and the assumptions involved when liquid media are employed.

Some comparisons of different solid media were then made and the results in Table 1 demonstrate the nature of the problem. Estimates on brain heart infusion agar are compared with Brewer's thioglycollate agar and with a mixture of equal volumes of these two media.

TABLE 1
DESTRUCTION OF *CL. BOTULINUM* L12 SPORES AT 100°C. COMPARISON OF THREE
AGAR MEDIA

Viable counts per ml. after 3 days at 37°C.

Time of Heating (min.)	Brain Heart Infusion Agar	Brewer's Thioglycollate Agar [*]	Brain Heart and Brewer's Agar [*] Mixed
0	54,000	29,000	53,000
4	52,000	26,000	46,000
8	38,000	21,000	45,000
16	32,000	19,000	38,000
32	6,600	10,000	15,000
64	80	1,100	670
128	< 1	2	1

^{*} Baltimore Biological Laboratories dehydrated product.

The estimates obtained on the three media vary considerably, but the relative performance of each is a function of the time of heating. Brain heart infusion agar gives a greater estimate of unheated spores, but the Brewer's thioglycollate agar indicates a greater survival after long periods of heating. The mixture of these two media tends to combine the advantages of each, and as a single medium for following the course of heat disinfection of *Cl. botulinum* spores it would be preferable to either of its constituent halves. Experiments were continued with the aim of obtaining some understanding of the factors which affect the phenomena just described, and of devising a thoroughly dependable medium for enumerating living spores of *Cl. botulinum*.

The effect of some of the constituents of Brewer's thioglycollate medium was tested in the following way. A medium was prepared from fresh pork

according to the formula given for the dehydrated product and this was compared with the brain heart infusion agar. Both media were compared with and without the methylene blue and thioglycollate as added to Brewer's dehydrated medium. Some results are shown in Tables 2 and 3.

TABLE 2

DESTRUCTION OF *CL. BOTULINUM* L12 SPORES AT 100°C. COMPARISON OF BRAIN HEART AND PORK INFUSION AGARS WITH BREWER'S THIOGLYCOLLATE AGAR*

Viable counts per ml. after 14 days at 25°C.

Time of Heating (min.)	Brain Heart Infusion Agar		Pork Infusion Agar		Brewer's Medium
	Without Thio-glycollate	0.01M Thio-glycollate	Without Thio-glycollate	0.01M Thio-glycollate	
0	80,000	108,000	55,000	87,000	35,000
40	100	16,000	7,500	19,000	7,700
80	< 1	70	48	350	70

* All media contain 2 p.p.m. methylene blue.

The results in Table 2 show that both the brain heart and pork infusion agars are superior to Brewer's medium for estimating unheated spores, and that the effects of added thioglycollate become relatively greater as the time of heating of the spores is increased.

TABLE 3

DESTRUCTION OF *CL. BOTULINUM* Q7 SPORES AT 100°C. COMPARISON OF AEROBIC AND ANAEROBIC INCUBATION

Viable counts per ml. after 14 days at 25°C.

Medium	Incubated in Air		Incubated in Hydrogen	
	0 min.	50 min.	0 min.	50 min.
Pork infusion agar	1,000	< 1	28,000	1
+ Methylene blue 2 p.p.m.	2,000	9	28,000	14
+ Na thioglycollate 0.01M	30,000	63	29,000	102
+ Methylene blue } + Na thioglycollate }	28,000	62	42,000	86

In Table 3 the effects of thioglycollate are shown with both aerobic and anaerobic incubation of pork infusion agar. It will be seen that, for unheated spores, the thioglycollate has increased the numbers of germinating spores only for aerobic incubation, but that the spores heated for 50 minutes showed a dependence on thioglycollate even when incubated in oxygen-free hydrogen for 14 days. Results of a number of experiments showed that methylene blue had no consistent effects of significance.

An apparent inconsistency between results in Tables 2 and 3 is perhaps worthy of comment. In Table 3 unheated spores of strain Q7 show very poor

development when incubated in air unless thioglycollate is added, whereas in Table 2 the figures for unheated spores of strain L12 show only small effects of thioglycollate in this medium. Such variation is typical when *Cl. botulinum* is incubated aerobically without thioglycollate at 25°C., rather than at 30 or 37°C. At the lower temperature there is a longer lag period before growth commences and during this time molecular oxygen may diffuse in amounts sufficient to render the medium unsuitable for germination of anaerobes. At 37°C. thioglycollate has little or no effect under these conditions, at 20°C. it is virtually indispensable, whereas at 25°C. the result is sensitive to minor experimental variations unless a reducing agent, such as thioglycollate, is present.

These and other experiments showed that several types of infusion media containing thioglycollate gave similar concordant estimates of unheated *Cl. botulinum* spores, but the variations between media were greater when inoculated with heated spores. Of the various media tested, pork infusion thioglycollate agar was generally, although not uniformly, the most satisfactory, whereas liver infusion agar was always unsatisfactory. Pork infusion thioglycollate agar was, therefore, selected as a standard basal medium and attempts to improve it further by the addition of supplements were continued.

(b) *The Effects of Adding Starch to Media*

In the course of testing various supplements in pork infusion thioglycollate agar, it was found that the addition of small amounts of starch increased the numbers of heated spores which produced colonies. The conditions under which the phenomenon has been observed are described briefly in the succeeding paragraphs. Although almost all experiments have been done with spores heated in phosphate buffer at pH 7.0, the phenomenon of increased spore germination in starch-supplemented media has been observed with spores heated in vegetable infusions and at various pH levels.

(i) *The Effect of Starch in Relation to the Duration of Heating of the Spores.*—The addition of starch has been found to produce relatively greater effects as the duration of heating of the spores is increased. Some typical results are given in Table 4. If the logarithms of the populations given in Table 4 are plotted against the time of heating, it will be seen that the destruction curves approximate to straight lines. For strain L12, the apparent rate of destruction shown by the starch-supplemented medium is about half that indicated by the starch-free medium.

(ii) *The Effects of Different Concentrations and Types of Starch.*—The effects of various concentrations of soluble starch in pork infusion thioglycollate agar are shown in Table 5. Results of a number of experiments have shown that maximum or near maximum counts are obtained with 0.1 to 0.2 per cent. soluble starch in the medium. The results with soluble starch have been obtained also with potato, maize, rice, and wheat starches, there being no suggestion of quantitative differences between starches at any of the concentrations tested. The effects of starch supplements were the same whether

the starch was added before autoclaving or by the aseptic addition of solutions separately sterilized by filtration or heat.

TABLE 4
DESTRUCTION OF *CL. BOTULINUM* SPORES AT 100°C. COMPARISON OF PORK INFUSION THIOGLYCOLLATE AGAR WITH AND WITHOUT 0.15 PER CENT. SOLUBLE STARCH
Viable counts per ml. after 14 days at 25°C.

Time of Heating (min.)	Strain L12		Strain 213B	
	Without Starch	With Starch	Without Starch	With Starch
0	19,000	19,000	250,000	270,000
10	17,000	21,000	57,000	81,000
20	6,700	7,200	6,500	10,500
40	2,000	4,000	80	450
60	100	1,000	1	44
100	4	110	< 1	1

(iii) *The Effects of Starch Supplements in Various Media.*—Similar responses to starch supplements have been observed in agar media based on infusions of pork, veal, calf brain, ox heart, and mutton. In all media the response to starch was restricted to heated spores, the estimates of unheated spores being independent of the starch supplement. An ox liver infusion agar gave satisfactory estimates of unheated spores, but was unsuitable for enumerating heated spores even when 0.1 per cent. starch was added.

TABLE 5
DESTRUCTION OF *CL. BOTULINUM* SPORES AT 100°C. EFFECT OF VARIOUS AMOUNTS OF SOLUBLE STARCH IN PORK INFUSION THIOGLYCOLLATE MEDIUM
Viable counts per ml. after 14 days at 25°C.

Strain	Time of Heating (min.)	Soluble Starch Added (% w/v)					
		5.0	1.0	0.1	0.01	0.001	0.000
L12	0	22,000	21,000	25,000	24,000	22,000	23,000
	60	90	95	73	18	7	2
Q7	0	57,000	65,000	70,000	68,000	67,000	65,000
	45	60	63	65	18	8	1
L4	0	21,000	23,000	18,000	19,000	18,000	18,000
	60	120	118	93	56	9	3

The effects of starch supplements were also found to be the same when pork infusion thioglycollate agar was solidified with 1.5 per cent. agar from four different sources. The samples, which were compared on a dry-weight basis, were of American (Difco), Japanese, Australian, and New Zealand origin.

Benefits of starch supplements were also demonstrated in media solidified with 15 per cent. gelatine, such media being incubated at 20°C.

Evidence that starch improves the performance of the liquid cooked meat medium as an agent for detecting heated *Cl. botulinum* spores was accumulated over several experiments. Comparisons were made with aliquots of the same heated suspension inoculated into a series of agar tubes and a series of cooked meat tubes with and without starch. In this way it was found, in a number of experiments, that all replicates (from 4 to 6) without starch failed to show growth, whereas all replicates containing 0.1 per cent. starch yielded growth of *Cl. botulinum*. The aggregate result of two or three such experiments satisfies the customary statistical criteria of significance. In several

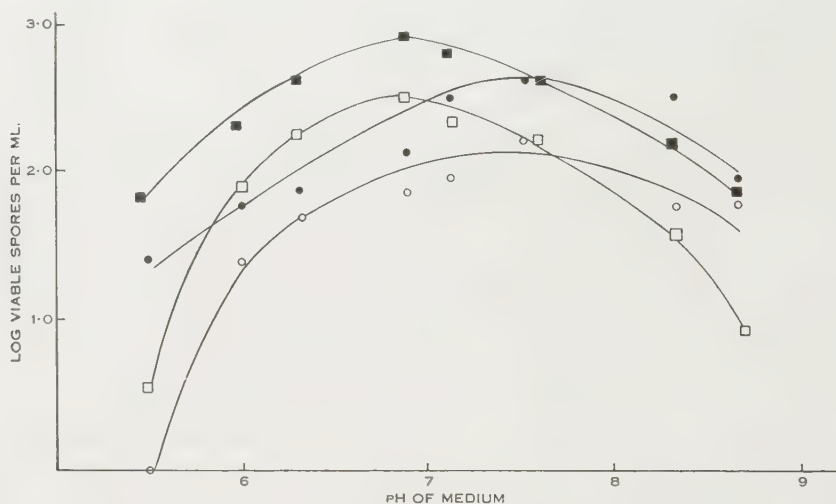


Fig. 1.—The effect of starch on the recovery of heated *Cl. botulinum* spores in pork infusion thioglycollate agar at various levels of pH.

● Strain 62A with, and ○ without 0.1 per cent. soluble starch in medium.
 ■ Strain 1787 with, and □ without 0.1 per cent. soluble starch in medium.
 Strain 62A—heated 60 min. at 100°C. Initial numbers 110,000 per ml.
 Strain 1787—heated 70 min. at 100°C. Initial numbers 21,000 per ml. Viable counts after 14 days at 25°C.

experiments the cooked meat medium (without starch) failed to detect surviving spores in 5 replicate samples each of 1 ml. when the solid media indicated up to 30 or 40 spores per ml. in starch-free media and from 90 to 120 spores per ml. when starch was present. Such experiments prove conclusively that the liquid cooked meat medium may be quite unsuitable for determining the presence or absence of viable spores in an inoculum of heated *Cl. botulinum* spores. The question whether or not the starch-supplemented cooked meat medium is adequate for detecting single survivors has not been investigated.

(iv) *The Effects of Starch in Relation to the pH of the Medium.*—The beneficial results of starch have been observed over a considerable range of pH, some typical results being given in Figure 1. The curves show that the ratio of the estimates on the starch-supplemented and control media varies only slightly with pH. It will be noted also that the optimum pH for germination of the heated spores is somewhat greater for strain 62A than for 1787. This result is typical of the differences observed between type A and type B strains, most, but not all, of the type A cultures showing the greatest germination of heated spores at pH 7.5 or above in contrast to an optimum of pH 7.0 or less for type B cultures. For unheated spores the effect of pH on the viable count is small between pH 6.0 and pH 8.5.

(v) *The Effects of Starch at Various Incubation Temperatures.*—Williams and Reed (1942) showed that incubation at 24 or 27°C. was more suitable for the detection of heated *Cl. botulinum* spores than incubation at 31 or 37°C. These findings have been confirmed in many experiments with cultures incubated at 20, 25, 30, and 37°C. For all strains, the greatest number of "survivors" was found with incubation at 25°C. Starch supplements gave increased numbers at all four temperatures, the absolute increase being greatest at 25°C.

(c) Experiments on the Mode of Action of Starch

Although it has been shown that several natural starches produced similar effects in promoting the germination of heated *Cl. botulinum* spores, this evidence did not exclude the possibility that the effects were due to minor constituents of the starch samples. It will now be shown that the observed activity is a feature of the undegraded starch molecule, especially of the straight-chain or amylose portion which has the capacity to adsorb substances which inhibit the germination of the heated spores.

TABLE 6
DESTRUCTION OF *CL. BOTULINUM* SPORES AT 100°C. EFFECT OF EXTRACTION OF STARCH
WITH ETHANOL AND ETHER
Viable counts per ml. after 14 days at 25°C.

Medium	Strain Q7		Strain L4	
	0 min.	45 min.	0 min.	60 min.
Pork infusion thioglycollate agar	39,000	4	11,000	3
+ 0.1% Soluble starch	44,000	75	13,000	42
+ 0.1% Ethanol extracted starch	38,000	70	11,000	42
+ Ethanol extract	38,000	3	16,000	1
+ 0.1 Ether-extracted starch	46,000	67	13,000	42
+ Ether extract	40,000	6	14,000	2

(i) *Purification Procedures.*—Starch was subjected to exhaustive extraction with selected polar and non-polar solvents as described by Schoch (1942a). Table 6 shows some results of an experiment using soluble starch extracted with 95 per cent. ethanol or diethyl ether. The extracts were concentrated to

an appropriate volume and added in equivalent concentration to the starch. Results of a similar nature have been obtained with maize starch extracted with methanol and carbon tetrachloride or treated by shaking in cold water.

The activity of starch was also found to be entirely unaffected by three successive precipitations in ethanol.

(ii) *Effects of Hydrolysed Starch.*—The activity of soluble starch was tested after enzymic and acid hydrolysis. A crude α -amylase preparation was prepared from pancreas and a crude β -amylase from wheat. Samples of starch treated with active or boiled enzyme were added to the medium prior to autoclaving. Results obtained with these two enzyme preparations and with takadiastase and hydrochloric acid hydrolysis are shown in Table 7.

TABLE 7
DESTRUCTION OF *CL. BOTULINUM* SPORES AT 100°C. COMPARATIVE EFFECTS OF STARCH
AND HYDROLYSED STARCH IN PORK INFUSION THIOGLYCOLLATE MEDIUM

Viable counts per ml. after 14 days at 25°C.

Medium	Strain L12		Strain 3149	
	0 min.	105 min.	0 min.	100 min.
Control	21,000	29	70,000	90
+ 0.1% Sol. starch	22,000	230	74,000	c.1000
+ 0.1% α -Amylase hydrolysed starch	24,000	50	78,000	185
+ 0.1% Starch + boiled α -amylase	20,000	230	72,000	c.1000
+ 0.1% β -Amylase hydrolysed starch	17,000	95	70,000	300
+ 0.1% Starch + boiled β -amylase	17,000	210	70,000	c.1000
+ 0.1% Starch hydrolysed by takadiastase	16,000	51	76,000	240
+ 0.1% Starch + boiled takadiastase	16,000	210	72,000	c.1000
+ 0.1% HCl hydrolysed starch	18,000	22	70,000	70

The smaller number of spores detected after enzymic hydrolysis is not due to inhibitory substances in the enzyme preparations, as the addition of the heat-inactivated enzymes has not impaired the action of the starch.

In other experiments it was found that glucose, maltose, and glucose-*l*-phosphate were all without effect on the numbers of colonies produced from a given inoculum. The glucose-*l*-phosphate was sterilized separately at pH 7.0 as a precaution against hydrolysis.

(iii) *The Effect of Synthetic Starch.*—A sample of synthetic starch, prepared and kindly donated by Dr. C. S. Hanes, was compared with soluble starch in two separate experiments. The first with type A strain L4 was done with pork infusion thioglycollate medium and the second with type B strain L12 in another batch of the same medium. Results are given in Table 8.

The synthetic starch evidently has an activity very similar to the soluble starch.

(iv) *Comparison of Amylose and Amylopectin Fractions.*—Samples of potato and maize starches were fractionated by the butanol solubility method of Schoch (1942*b*), the maize starch being first extracted with methanol. The

activity of the butanol-insoluble (amylose) and butanol-soluble (amylopectin) fractions was compared with the parent starch. Some typical results are shown

TABLE 8

DESTRUCTION OF *CL. BOTULINUM* SPORES AT 100°C. COMPARISON OF SOLUBLE AND SYNTHETIC STARCHES

Viable counts per ml. after 14 days at 25°C.

Medium*	Strain L4		Strain L12	
	0 min.	60 min.	0 min.	120 min.
Pork infusion thioglycollate agar	12,000	20	21,000	15
+ 0.1% Soluble starch	14,000	104	22,000	c.200
+ 0.01% Soluble starch	15,000	49	20,000	73
+ 0.1% Synthetic starch	18,000	100	19,000	c.200
+ 0.01% Synthetic starch	12,000	52	22,000	80

* Batch No. 45 used with strain L4 and batch No. 46 with strain L12.

in Figure 2. The figures for unheated spores are not shown but, as in all other experiments with *Cl. botulinum*, the estimates of unheated spores were not affected by the presence or absence of starch in the medium.

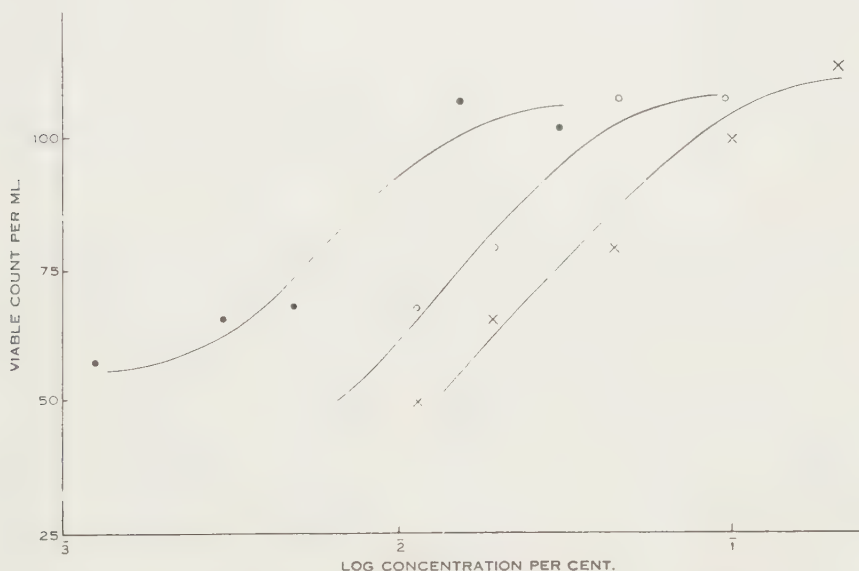


Fig. 2.—The effects of amylose and amylopectin fractions of starch on the estimates of heated *Cl. botulinum* Q7 spores in pork infusion thioglycollate agar. O Potato starch. ● Amylose (butanol-insoluble) fraction. X Amylopectin (butanol-soluble) fraction. Initial population spores 41,000 per ml. heated for 60 min. at 100°C. Viable counts after 14 days at 25°C.

Inspection of the curves in Figure 2 shows that the butanol-insoluble (amylose) fraction is some four to five times as active as the potato starch,

whereas the butanol-soluble (amylopectin) fraction was the least active. Results of a very similar nature were obtained with maize starch fractions. As the reported amylose contents of potato and maize starches are of the order of 22 per cent. (Schoch 1942*b*), the results are consistent with the view that the amylose fraction accounts for the activity of natural starches. As the butanol-soluble fractions were not free from amylose, it is possible that such activity as they had was due to their amylose content.

(v) *Comparison Between Starch and Other Substances.*—Of many substances tested, only activated charcoal and serum albumin had any activity comparable with that shown by starch. The following substances were tested in 0.1 per cent. concentration and found to be without effect on the number of colonies developing from an inoculum of heated spores: fructose, sucrose, lactose, cellobiose, trehalose, melezitose, raffinose, inulin, gum acacia, gum mastic, blood group A polysaccharide, glycogen, yeast extract, and talc. Two samples of glycogen were tested, one being prepared from oysters and the other from dog liver. Both were inactive at 0.1 and 0.5 per cent. concentrations, and their presence did not depress the effects of starch. Their lack of activity is not, therefore, due to inhibitors present as impurities in the preparations, but is in harmony with the finding that the properties of starch are associated with the straight-chain structure of amylose.

Although the evidence quoted in the preceding paragraphs suggests a physico-chemical adsorption as the most likely reason for the activity of starch, there is no critical proof that this is, in fact, the explanation. More convincing evidence is given in Table 9, which shows the comparative effects of starch,

TABLE 9
DESTRUCTION OF *CL. BOTULINUM* SPORES AT 100°C. COMPARISON OF STARCH, CHARCOAL,
AND SERUM ALBUMIN ADDITIONS TO PORK INFUSION THIOGLYCOLLATE AGAR
Viable counts per ml. after 14 days at 25°C.

Medium	Strain Q7		Strain 213B	
	0 min.	40 min.	0 min.	50 min.
Pork infusion thioglycollate agar	52,000	20	980,000	24
+ 0.1% Starch	65,000	520	1,140,000	490
+ 0.1% Charcoal	49,000	360	1,100,000	450
+ 0.1% Serum albumin	52,000	340	1,020,000	320
+ 0.1% Starch	49,000	530	1,080,000	410
+ 0.1% charcoal				
+ 0.1% Starch	61,000	410	980,000	390
+ 0.1% serum albumin				

charcoal, or bovine serum albumin in pork infusion thioglycollate agar. The albumin, prepared by the method of McMeekin (1940), was sterilized by filtration and added to the sterile agar at 50°C. to prevent denaturation. The results show that all three substances produce similar effects, the combined effects of starch and charcoal, or of starch and albumin, being no greater than those of

starch alone. It is most likely, therefore, that each substance increases the germination of heated spores by virtue of its capacity for adsorption, the most likely explanation being an adsorption of substances which inhibit spore germination.

Following the reports of Kodicek and Worden (1945) that cholesterol reversed the anti-bacterial effects of unsaturated fatty acids against *Lactobacillus helveticus*, some tests were carried out with 0.1 per cent. cholesterol incorporated in the medium. The results were not consistent, but in one experiment the cholesterol did increase the numbers of colonies of *Cl. botulinum* which developed. The cholesterol was, however, less effective than an equal amount of soluble starch. In other experiments the cholesterol supplement was without effect. It would seem, therefore, that either cholesterol is less efficient than starch in protecting the spores against inhibition by unsaturated fatty acids, or inhibition by other substances is involved.

IV. RESULTS OF EXPERIMENTS WITH OTHER SPECIES OF CLOSTRIDIUM

Starch in 0.1 per cent. concentration was found to increase the numbers of colonies developing in pork infusion medium inoculated with heated spores of the following organisms: *Cl. histolyticum*, *Cl. oedematiens*, *Cl. bifermentans*, *Cl. botulinum* type C β , *Cl. tetani*, *Cl. sporogenes*, *Cl. thermosaccharolyticum* N.C.A. No. 3814, and *Clostridium* sp. N.C.A. No. 3679. Some of the above types were relatively heat-sensitive, and for the first four organisms listed most spores were destroyed in less than 20 minutes at 100°C. The two last-mentioned strains are used as test organisms by the N.C.A. Research Laboratories, and produce spores which are highly resistant to heat.

V. DISCUSSION

It is clear that the spores of *Cl. botulinum* "surviving" heat treatments which have destroyed most of their number are much more difficult to cultivate and enumerate than the spores present prior to heating. This is manifest in several ways. For instance, the estimates of heated spores show, in comparison with those for unheated spores, a greater dependence on reducing agents such as thioglycollate, a more marked optimum temperature, a more pronounced dependence on pH, and a greatly increased sensitivity to inhibitors adsorbable on starch, charcoal, or serum albumin. At present it is not known why the environment is more critical for the heated spores. One possibility is that, as a result of heating, the surviving spores require altered cultural conditions for germination. Another possibility is that the optimum conditions are the same for both unheated and heated spores, but for heated spores the probability of germination diminishes more rapidly as one departs from the optimum. In other words, the range of conditions permitting germination may become more circumscribed with increasing "heat injury" of the spores.

If the ideal conditions for the germination of heated spores of a culture of *Cl. botulinum* were known, then a true estimate of the spores surviving a

given heat treatment could be obtained by inoculating the spores into a medium providing the appropriate conditions. The accuracy of such estimates would be affected only by sampling errors. At present, however, these ideal conditions are not known and one can only compare estimates under various conditions and assume that the greatest estimates approximate most closely to the true value.

In the present experiments the largest numbers of survivors were generally found in a pork infusion thioglycollate agar adjusted to the most favourable pH for the particular strain, supplemented with a suitable adsorbent, such as starch, and incubated at 25°C. This is not proposed as an ideal medium, but it has proved more efficient than those described previously. The ability of such a medium to detect as many as 100 spores, which are unable to germinate in the commonly used cooked meat medium, provides clear evidence of the unsuitability of the latter for detecting heated *Cl. botulinum* spores. It need not be emphasized that errors of this magnitude are important in experiments with spores of this toxigenic organism.

Starch has been shown to increase the estimates of heated spores under a wide range of conditions and there can be no doubt that it acts, like charcoal and serum albumin, by adsorbing inhibitory substances from the medium. The results provide no evidence regarding the nature of the inhibitors although it is very probable that unsaturated fatty acids are involved. These substances are of widespread occurrence and have been reported to inhibit the growth of several different types of bacteria (Kodicek and Worden 1945; Ley and Mueller 1946; Pollock 1947). Recently Wynne and Foster (1948) have confirmed the effect of starch previously reported by Olsen and Scott (1946), and Foster and Wynne (1948) have shown that the germination of *Cl. botulinum* spores is inhibited by traces of long chain unsaturated fatty acids, the inhibition being prevented by the incorporation of starch in the medium. This finding establishes a reason for the effect of starch, but it still remains to be determined whether unsaturated fatty acids are the principal inhibitors in the media or whether other substances are involved.

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THE ENUMERATION OF HEATED BACTERIAL SPORES

II. EXPERIMENTS WITH BACILLUS SPECIES

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Summary

Experiments with 13 strains of *Bacillus* isolated from canned foods have shown that, like *Clostridium* species, the germination of heated spores is affected by inhibitors present in the media. As with *Clostridium* species, susceptibility to inhibitors increases with the amount of heating to which the spores are exposed. The *Bacillus* strains, however, show more variation in the extent to which different strains are inhibited in a particular medium. There is also evidence of substantial differences in the amounts or types of inhibitors which are contained in different media. Some of the differences between media are due to variations in the concentrations of inhibitors which are adsorbed on charcoal, starch, or serum albumin, but some of the differences are attributable to inhibitors which are not adsorbed on charcoal. Treatment of nutrient agar with charcoal and subsequent removal of the charcoal is as effective in removing inhibitors as incorporation of the charcoal in the medium. The medium, therefore, is the principal or sole source of inhibitors. Attempts to demonstrate inhibitors in the inoculum were unsuccessful. It is unlikely that unsaturated fatty acids account for more than part of the observed inhibition. A suitable adsorbent should be incorporated in media used for evaluating the thermal destruction of *Bacillus* spores.

I. INTRODUCTION

In a previous paper (Olsen and Scott 1950) it was shown that the spores of several species of *Clostridium*, including *Cl. botulinum*, became progressively more sensitive to traces of inhibitory substances as the amount of lethal heating to which they had been subjected was increased. The number of heated spores capable of germinating and forming colonies increased when suitable adsorbents such as starch, charcoal, or serum albumin were incorporated in the nutrient medium. In this paper it will be shown that a similar phenomenon applies to spores produced by a number of *Bacillus* strains which have been isolated from spoiled canned foods. It will also be shown that the inhibitors concerned are derived mainly, if not entirely, from the various laboratory media commonly used for the cultivation and enumeration of these organisms.

II. METHODS

The methods generally were similar to those described in the previous paper (Olsen and Scott 1950) except for the following modifications. Spore suspensions were usually harvested from surface growths on various agar media.

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In the later experiments the temperatures of the oil bath were controlled more accurately, the nominal temperatures being realized within 0.05°C . Viable counts were made with the usual plating techniques, care being taken to observe the precautions described by Wilson *et al.* (1935). In addition to the media described in the previous paper, nutrient agar and dextrose tryptone agar were used. These were prepared according to the formulation given in the Difco Manual (1944).

The organisms studied included 10 strains isolated in these laboratories from spoiled canned foods and designated A21, A65, 196, 209, 256, 262, 320, 414, 1057, and 1541. Three strains of American origin (698, 1518, and HAX) were obtained through the courtesy of Dr. E. J. Cameron, N.C.A. Research Laboratories, Washington, U.S.A.

The activated charcoal used was a commercial product of animal origin, and the bovine serum albumin was partially purified by ammonium sulphate fractionation as described by McMeekin (1940). The albumin solution was dialysed against phosphate buffer, and additions of Seitz filtered material made in terms of the dry weight of protein in the solution.

III. RESULTS

(a) *Experiments with Different Strains on Various Media*

As with *Clostridium* spores, the apparent susceptibility to inhibitors increases with the amount of heating to which the spores are exposed. A striking example of this appears in Figure 1, which shows results for *Bacillus* No. 262 grown on brain heart infusion agar. The only factor which contributes to the two widely different rates of destruction is that in one case the nutrient medium contained a supplement of 0.2 per cent. of soluble starch. The supplemented and control media both gave similar estimates of the spores which were viable before heating, but as the duration of heating and the associated spore mortality increased, the unsupplemented control medium became progressively less suitable for estimating survivors. It is perhaps worthy of comment that the highly erroneous data obtained with the control medium still approximate to a straight line when the logarithm of the number of survivors is plotted against the time of heating. Obviously arguments based on conformity to this type of destruction curve cannot be accepted as an indication that the data are reliable.

The various *Bacillus* strains tested have shown considerable differences in their susceptibility to inhibitors, some, like No. 262, showing large increases when adsorbents such as starch were incorporated in the medium, whereas others, such as the National Canners' Association test organism *Bacillus* No. 1518, show only a small response. All 13 strains tested have, however, shown consistent evidence of increased germination of heated spores when inhibitors were removed by starch or charcoal. Some strains have shown a marked response to thioglycollate as well as to starch, and for some organisms the combined effects of thioglycollate and starch have been more than additive. Some

results obtained with four strains inoculated into four basal media are given in Table 1. Comparisons are based on aliquots of the same spore suspensions and of the same batch of basal medium.

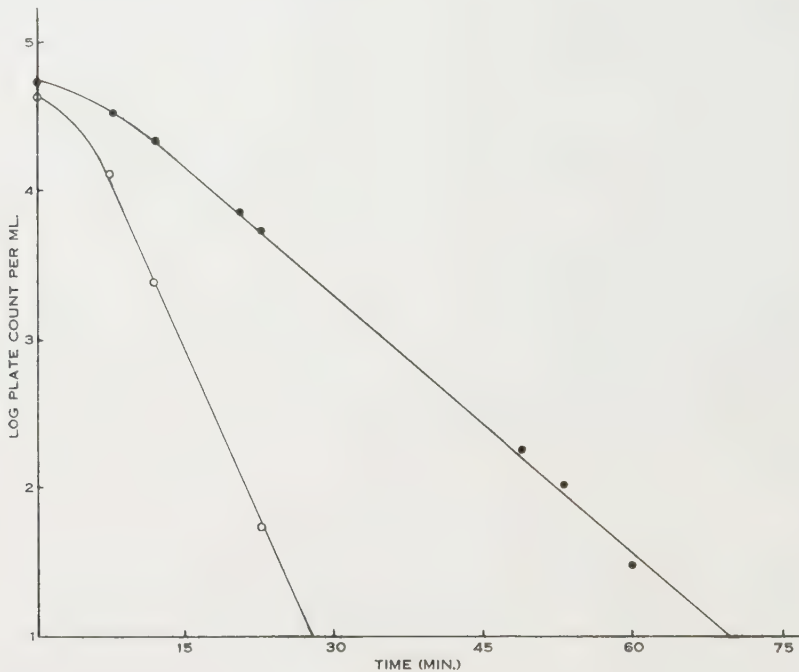


Fig. 1.—Destruction of *Bacillus* No. 262 spores in M/15 phosphate buffer, pH 7.0, at 115°C. as measured on brain heart infusion agar with and without 0.2 per cent. soluble starch.

O Medium without starch. ● Medium with 0.2 per cent. added starch. Plates incubated for 2 days at 55°C.

It is evident from the data in Table 1 that there are considerable differences between strains and between media. All strains reveal some increases due to the starch supplement, especially on the more complex infusion media. The effects are greatest with strain 262 and least with strain 1518. The effect of thioglycollate is more variable, being marked for strain 262, small for strain 1518, and absent for strains 320 and A21. The addition of both starch and thioglycollate results in remarkable increases for strain 262, a small response for strain 1518, and no increase over the values for starch alone with the remaining two organisms.

(b) Comparative Effects of Starch, Charcoal, and Serum Albumin

The addition to the medium of starch, charcoal, or serum albumin has been shown by Pollock (1947) to promote the growth of *Haemophilus pertussis* and by Olsen and Scott (1950) to increase the germination of heated *Clostridium* spores. Tests were made of the comparative effects of these three substances on the germination of *Bacillus* spores, and the results with strain 320 are given in Table 2.

TABLE I
RESPONSE OF FOUR STRAINS OF *BACILLUS* TO VARIOUS SUPPLEMENTS ON FOUR BASAL MEDIA
Figures represent plate counts per ml. after 2 days at 50°C. for strains 1518, 262, and 320 and after 2 days at 30°C. for strain A21

Basal Medium	Supplement ^a	Strain 1518		Strain 262		Strain 320		Strain A21	
		0 min.	35 min. at 115°C.	0 min.	60 min. at 115°C.	0 min.	12 min. at 115°C.	0 min.	40 min. at 100°C.
Nutrient agar pH 7.9	A	97,000	44	3,000	< 1	28,000	25	6,800,000	90
	B	101,000	59	8,000	225	39,000	74	7,500,000	137
	C	94,000	71	6,000	194	39,000	24	6,700,000	59
	D	112,000	104	8,000	1200	67,000	98	4,700,000	24
Brain heart infusion agar pH 7.8	A	—	32	13,000	76	20,000	4	9,000,000	27
	B	—	61	9,000	700	24,000	37	7,500,000	152
	C	—	51	11,000	710	13,000	6	7,500,000	24
	D	—	93	13,000	2000	34,000	30	7,800,000	90
Pork infusion agar pH 8.0	A	50,000	< 1	2,000	1	39,000	57	8,200,000	91
	B	50,000	< 1	5,000	100	68,000	100	7,800,000	108
	C	52,000	15	5,000	19	98,000	54	8,500,000	110
	D	50,000	72	10,000	705	93,000	78	8,400,000	98
Dextrose tryptone agar pH 7.9	A	142,000	64	9,000	128	15,000	3	8,100,000	20
	B	138,000	77	10,000	680	19,000	24	7,900,000	85
	C	138,000	78	12,000	650	12,000	6	8,600,000	12
	D	128,000	90	14,000	1250	19,000	25	8,700,000	76

^a Supplements added: A—none; B—0.1% soluble starch; C—0.01M Na thioglycollate; D—0.1% starch + 0.01M Na thioglycollate.

All three substances increase the numbers of heated spores which form colonies and, if this is expressed in terms of the apparent rate of destruction, the supplemented media indicate significantly lower rates of death than does the control medium. There is a suggestion that, in the amount added, charcoal may be slightly more effective than starch, but there is no indication

TABLE 2

EFFECT OF SOLUBLE STARCH, SERUM ALBUMIN, AND ACTIVATED CHARCOAL ON THE GERMINATION OF HEATED *BACILLUS* SPORES (STRAIN 320) IN BRAIN HEART INFUSION AGAR
pH 6.70 $\pm$ 0.03

Figures represent plate counts per ml. after 5 days at 50°C.

Medium Supplement	Time of Heating at 110°C.					K110°†	S.D.
	0 min.*	5 min.	20 min.	40 min.	60 min.		
None	1,400	32,000	1,420	56	< 1	0.0786	0.0045
Starch 0.1%	1,800	34,000	4,600	400	23	0.0567	0.0014
Albumin 0.2%	2,600	34,000	—	500	35	0.0532	0.0015
Charcoal 0.2%	2,850	30,000	5,000	870	72	0.0464	0.0021
Starch 0.1% + albumin 0.2%	3,000	50,000	3,700	510	50	0.0520	0.0042
Starch 0.1% + charcoal 0.2%	4,300	38,000	4,600	560	44	0.0522	0.0020
Albumin 0.2% + charcoal 0.2%	2,300	34,000	5,200	540	61	0.0505	0.0010
Starch 0.1% + albumin 0.2% + charcoal 0.2%	—	—	4,600	490	54	0.0483	0.0002

* Viable count at 0 min. low due to dormancy, maximum count being reached after approximately 5 min. at 110°C.

† Figures for 0 min. omitted in calculation of rate of destruction. Differences necessary for significance, 0.0076 ($p = 0.05$) and 0.0106 ($p = 0.01$). In the calculation of K and its error the mean count at each time was weighted in proportion to the number of replicates on which it was based.

that the combined effects of any pair or of all three substances are any greater than the effect of any one of the adsorbents. The indication is, therefore, that starch, charcoal, and serum albumin all act in a similar way by adsorbing from the medium the same type(s) of inhibitory substance(s).

(c) The Response to Charcoal Supplements added to Different Basal Media

If a number of media are all nutritionally adequate for spore germination and growth of a particular organism, any variation in their ability to promote the germination of spores may well be due to variations in the concentration and type of sporostatic substances which they contain. If these inhibitory

substances are capable of adsorption on charcoal, then it might be expected that a sufficient concentration of added charcoal would bring each medium to the same level of efficiency in promoting spore germination. This question was examined with *Bacillus* No. 320 and the results of adding various amounts of charcoal to five basal media are shown in Figure 2.

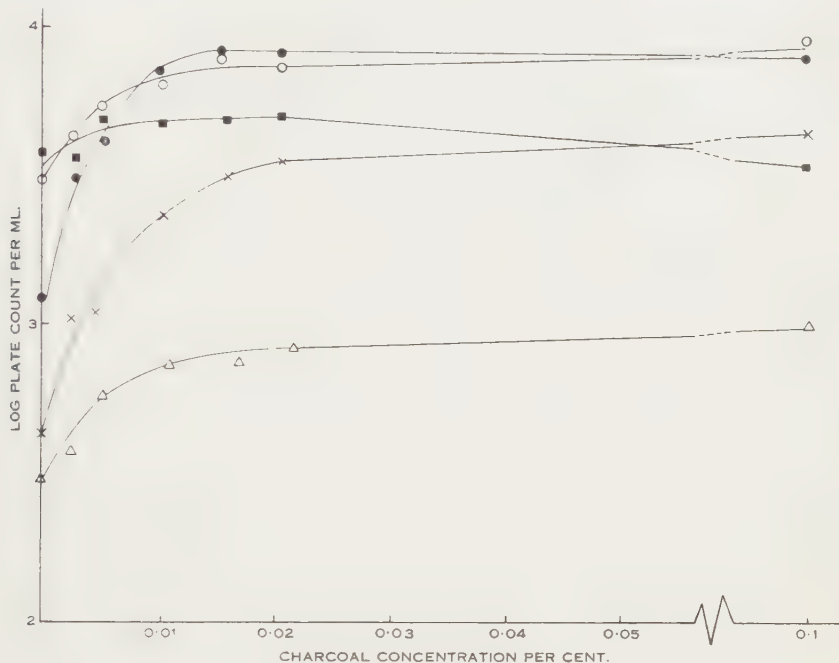


Fig. 2.—Effect of different concentrations of charcoal added to five media on the estimates of *Bacillus* No. 320 spores after 31 minutes at 110°C.

○ Nutrient agar. Mean pH 6.6. ● Pork infusion agar. Mean pH 6.4.
 ■ Dextrose tryptone agar. Mean pH 6.5. X Brain heart infusion agar. Mean pH 6.3. △ Peptic digest agar. Mean pH 6.4. Plates incubated 5 days at 50°C. Initial population 35,000 per ml. approx.

The curves show how the estimates of the spores surviving heating for 31 minutes at 110°C. increase with increasing charcoal concentration in each medium. It will be noted that only two of the five media, namely nutrient agar and pork infusion agar, show signs of reaching the same maximum estimate in the presence of charcoal. For the other three media it is clear that even greater concentrations of charcoal will not cause the same number of spores to germinate and produce colonies. It is of interest to note that in the absence of added charcoal the two comparatively simple media, nutrient agar and dextrose tryptone agar, gave considerably greater estimates than the more complex infusion and peptic digest media. The improvement following the addition of charcoal was, however, greatest for nutrient agar and pork infusion agar, least for dextrose tryptone agar, and intermediate for brain heart infusion. The peptic digest medium proved quite unsuitable for enumerat-

ing viable spores of this organism even when supplemented with 0.1 per cent. of charcoal. It may be added that all media except the peptic digest gave comparable estimates of the spores viable prior to heating.

The failure of the charcoal supplement to equalize the performance of the different media would seem to be most probably due to the presence of inhibitory substances which are not adsorbed by charcoal. The alternative that complex media such as brain heart infusion or peptic digest medium are lacking in essential nutrients seems less likely in view of the ability of these media to support the growth of a great variety of bacteria with complex nutritional requirements.

(d) The Removal of Inhibitors from Media by Adsorption with Charcoal

If the adsorbents are able to bind the inhibitory substances sufficiently firmly to prevent their anti-bacterial action it should be possible to remove the adsorbent with the inhibitors still attached. This has been examined with brain heart infusion and nutrient agars treated with activated charcoal.

In the first experiment the brain heart infusion agar, with and without 0.2 per cent. charcoal, was compared with a medium prepared by treating the double strength broth moiety with 0.4 per cent. charcoal, removing the charcoal by filtering the autoclaved mixture through sintered glass, and aseptically adding the filtered broth to the separately autoclaved agar. Appropriate control treatments with the charcoal remaining in the medium were included. The results are shown in Table 3. The estimates of surviving spores indicate different apparent rates of destruction depending on the medium used. The medium from which the charcoal was removed (*E*) shows a lower death rate than the appropriate control (*C*), but higher than when the charcoal remained in the medium (*D*). There is no suggestion that separate autoclaving of the broth and agar fractions has had any significant effect on the properties of the medium (treatments *A* and *C*; *B* and *D*).

The second experiment was carried out with nutrient agar to which was added 0.2 per cent. charcoal either before or after autoclaving. Portions of each medium were centrifuged under sterile conditions at 50°C., and the charcoal-free supernatant compared with the uncentrifuged portion with the charcoal remaining in the medium. The control medium received no treatment with charcoal. Table 4 summarizes the results, which show that all four media treated with charcoal gave significantly lower "rates of destruction" than the control medium. There is no difference between the media from which the charcoal was removed by centrifuging and those in which it remained, indicating that the medium is the principal source of the inhibitors adsorbable on charcoal. That the charcoal did, in fact, adsorb a considerable quantity of material was shown by washing the charcoal precipitate in boiling water, drying at 110°C., and reweighing. The 2.00 g. of dry charcoal originally added to one litre of medium had increased to 2.61 g. After extraction with dilute HCl and NaOH the dry weight was 2.33 g. and this was further reduced to 1.98 g. after 24 hours' extraction with methanol.

Comparison of the experiments in Tables 3 and 4 provides further confirmation of the superiority of the nutrient agar for enumerating spores of *Bacillus* No. 320, the apparent rate of destruction when this medium is the index of survival being less than half the rate shown by brain heart infusion agar. This is, however, partly due to the difference in pH of the media, the value of pH 6.4 for the nutrient agar being close to the optimum for this strain.

TABLE 3
EFFECT OF CHARCOAL ADSORPTION OF BRAIN HEART INFUSION AGAR ON THE
ESTIMATES OF HEATED SPORES OF *BACILLUS* NO. 320

Figures represent plate counts per ml. after 5 days at 50°C.

Treatment of Medium*	Time of Heating at 110°C.					K _{110°} †	S.D.
	0 min.‡	10 min.	20 min.	30 min.	40 min.		
A Brain heart infusion agar control	860	3,100	660	111	44	0.0632	0.0035
B Brain heart infusion agar + 0.2% charcoal	2,180	12,300	4,000	1,600	430	0.0480	0.0017
C Brain heart broth + agar (mixed after autoclaving)	920	5,000	1,250	300	51	0.0659	0.0023
D Brain heart broth with 0.4% charcoal + agar (mixed after autoclaving)	1,790	8,400	2,960	930	310	0.0477	0.0008
E Brain heart broth with 0.4% charcoal removed + agar (mixed after autoclaving)	960	7,700	2,140	560	136	0.0569	0.0011

* Media A and B: pH 7.3; C, D, and E: pH 7.5.

‡ Viable count at 0 min. low due to dormancy, maximum count being reached after approximately 5 min. at 110°C.

† Figures for 0 min. omitted in calculation of rate of destruction. Differences necessary for significance, 0.0066 ($p = 0.05$) and 0.0095 ($p = 0.01$). In the calculation of K and its error the mean count at each time was weighted in inverse proportion to its variance.

It is also evident that adsorption of the complete nutrient agar medium and removal of the charcoal by centrifuging has been more effective in removing inhibitors than adsorption of the broth moiety of brain heart infusion. This may be due to the greater concentration of inhibitors in brain heart infusion (see Fig. 2), or to the agar itself being a significant source of inhibitors, as was

found by Ley and Mueller (1946) in experiments with the gonococcus. These authors found that exhaustive methanol extraction effectively removed inhibitors of a fatty acid nature from the agar.

TABLE 4
THE EFFECT OF CHARCOAL ADSORPTION OF NUTRIENT AGAR ON THE ESTIMATES
OF HEATED SPORES OF *BACILLUS* NO. 320

Figures represent plate counts per ml. after 5 days at 50°C. pH of media 6.40 ± 0.05

Treatment of Medium	Time at 110°C.			K110°*	S.D.
	6 min.	31 min.	56 min.		
A Nutrient agar control	23,500	3,740	565	0.0323	0.0002
Nutrient agar +					
B 0.2% charcoal (added before autoclaving)	33,600	18,400	4,760	0.0166	0.0038
Nutrient agar +					
C 0.2% charcoal (added after autoclaving)	34,600	19,000	4,970	0.0188	0.0033
As for B, but char- coal removed be- fore using medium	37,000	18,000	5,340	0.0168	0.0014
As for C, but char- coal removed after 3 hours at 50°C.	36,600	19,200	5,160	0.0165	0.0033

* Differences necessary for significance, 0.0102 ($p = 0.05$) and 0.0160 ($p = 0.01$). In the calculation of K and its error the mean count at each time was weighted in inverse proportion to its variance.

If simple synthetic media are prepared and solidified with methanol-extracted agar it should be possible to produce inhibitor-free media in which starch or charcoal supplements would be without effect. Experiments in this direction have been unsuccessful, considerable effects of starch or charcoal still being observed in media containing only simple substances and methanol-extracted agar. In these synthetic media some samples of charcoal were markedly toxic, this being presumably due to inhibition by metals in the charcoal as the toxic effects were reduced by extraction with mineral acid or 8-hydroxyquinoline, or by incorporating 1.0 p.p.m. of the latter substance in the medium. Charcoal prepared from sucrose lacked this toxic property.

The possibility exists that the inoculum itself is a significant source of inhibitors, but treatment of heated spore suspensions by exposing them to charcoal in cellophane sacs or by mixing with charcoal and subsequent removal

of the adsorbent has failed to show any inhibitors in the inoculum. Such evidence is, however, of a negative character and does not eliminate the possibility that the heated spores themselves may, at times, carry substances which are inimical to their germination.

IV. DISCUSSION

The experiments provide further evidence that there are considerable technical difficulties in the detection and enumeration of *Bacillus* spores that have been subjected to lethal conditions of heating. Some of the observations are without any obvious explanation and simply raise questions without solving them. It is believed, however, that such effects as the response to combined supplements of thioglycollate and starch (Table 1) are of sufficient magnitude to establish their importance, even as unexplained facts.

It is clear that, as the period of heating is increased, the surviving spores become increasingly sensitive to inhibitors in the medium. It is not known, however, whether this is due to a progressive "heat injury" which causes the spores to become more sensitive to inhibitors, or whether the most heat-resistant spores in a suspension are inherently the most susceptible to inhibitors. Whatever the cause, however, the fact remains that the addition of a suitable adsorbent to a particular medium may cause it to indicate a significantly lower rate of destruction than that revealed by the control, unsupplemented medium. In general, the unheated spores show little or no evidence of susceptibility to inhibitors, at least in the concentrations in which they occur in common media. This is true even for organisms such as *Bacillus* No. 320 where only 2 to 5 per cent. of the unheated spores may germinate unless the spores are subjected to an appropriate "heat activation" treatment.

That starch, charcoal, and serum albumin owe their effects to adsorption of inhibitors is shown by the fact that all three substances produce similar effects when added singly or in combination, by the shape of the curves relating the response to the concentration of added charcoal, and by the removal of inhibitors from the medium along with the charcoal. The latter result shows also that the medium is, in fact, the principal source of the inhibitors. Although the various media all show evidence of inhibitors which are adsorbed on charcoal, the results shown in Figure 2 rather strongly suggest that some media contained inhibitors which were not adsorbed.

The present experiments furnish no evidence regarding the nature of the inhibitors although it is probable that long chain unsaturated fatty acids are involved. Traces of these acids have been shown by a number of workers to inhibit the growth of a variety of organisms including species of *Haemophilus*, *Lactobacillus*, *Mycobacterium*, and *Neisseria*, the inhibition being reversed by one or more of the adsorbents used in these experiments. Wynne and Foster (1948) and Foster and Wynne (1948) have shown that germination of the spores of *Cl. botulinum* was inhibited by traces of unsaturated fatty acids although the growth of vegetative cells was unaffected by much higher concentrations. These authors found no inhibition of the germination of unheated

spores of four species of *Bacillus* by unsaturated fatty acids. Murrell (unpublished data) has, however, found that the susceptibility of *Bacillus* spores to unsaturated fatty acids increases with the duration of heating. Although it is likely that these fatty acids do account for at least part of the inhibitory properties of the media, more direct evidence is needed to demonstrate whether or not they are the only substances of importance. In this connexion it should be remembered that starch, charcoal, and serum albumin are each able to combine with many substances other than fatty acids. Schuhardt *et al.* (1949) have recently reported that certain peptones contain an anti-brucella factor which can be adsorbed on charcoal. The substance is not a fatty acid, but is believed to be an oxidized polypeptide or amino acid.

It is obvious that many important problems relating to the germination of heated spores still remain unanswered. Studies of spore germination in a chemically defined, inhibitor-free environment should enable the optimum conditions for germination to be defined, and lead to more reliable and precise techniques for evaluating rates of thermal destruction. Meanwhile, there is need for considerable care in selecting a suitable medium for these studies, and it would seem advisable to supplement the medium with a suitable adsorbent and to confirm the estimates with at least one other basal medium.

V. ACKNOWLEDGMENT

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THE RESPIRATORY METABOLISM OF HELMINTHS

By MARIAN LAZARUS*

[Manuscript received January 5, 1950]

Summary

The respiratory metabolism of the trematode *Paramphistomum cervi* and the nematodes *Haemonchus contortus*, *Ostertagia circumcincta*, *Syphacia* sp., *Heterakis spumosa*, *Strongylus equinus*, and *Strongylus vulgaris* have been examined.

The nematodes gave Q_{O_2} values ranging from -7.4 to -1.1 . When calculated on the basis of the relative surface areas of the parasites the differences in oxygen uptake were much smaller. Cyanide inhibited the uptake of oxygen; R.Q. values ranged from 0.8 to 3.3. Nematodes inhabiting those parts of the gut in which bacterial fermentation is most active generally showed higher R.Q. values.

Paramphistomum cervi gave the low Q_{O_2} of -0.03 , and an R.Q. of 8 or over. Cyanide caused a pronounced increase in the uptake of oxygen. When the trematodes were incubated aerobically *in vitro* the R.Q. gradually fell and the activating effect of cyanide decreased. The cyanide activation of oxygen uptake of brei prepared from *Paramphistomum cervi* was not obtained when methylene blue was present. The present investigation has shown that the pattern of respiratory metabolism in helminths may vary, even among morphologically related forms. However, different species of parasites from regions of similar physiological activity in the host show some similarity in the nature of their respiratory activity.

I. INTRODUCTION

The respiratory metabolism of large helminths such as *Ascaris lumbricoides* and *Parascaris equorum* has been examined extensively (see Laser 1944 for references) but smaller parasites, particularly oxyurids, have not been examined to any extent. Further, most of the species already examined have been parasites of the small intestine. The present investigation includes species which, though not closely related taxonomically, come from somewhat similar physiological regions in the host's alimentary tract. Thus the oxyurid parasites *Heterakis spumosa* and *Syphacia* sp., and the strongylids *Strongylus equinus* and *S. vulgaris* are found in the colon of rats and mice and the caecum of horses, respectively, both of which are regions of active bacterial fermentation. The trematode *Paramphistomum cervi* was obtained from the reticulum of the ox, where bacterial fermentation takes place also. The other strongylids, *Haemonchus contortus* and *Ostertagia circumcincta*, were obtained from the abomasum of the sheep where bacterial fermentation does not usually occur.

The selection of parasites might be expected to show whether the pattern of their respiratory activity is determined more by taxonomic relationships based on morphological considerations, or by the nature of their environments.

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II. METHODS

Syphacia (probably *S. obvelata*) and *Heterakis spumosa* were collected from the colon of mice and rats which had been fasted overnight. The parasites were washed repeatedly in 0.9 per cent. saline till clean, the whole process taking 2-5 hours. *Haemonchus contortus* and *Ostertagia circumcincta* were recovered from the abomasum of sheep and used about three to six hours after the killing of the host. *Strongylus equinus* and *S. vulgaris* were collected from the caecum of horses about four hours after the death of the host and examined about $\frac{3}{4}$ hour later. The stomach fluke of cattle, *Paramphistomum cervi*, found adhering by the posterior sucker to small papillae on the reticulum, was removed two to five hours after the death of the host and used at periods of $\frac{1}{4}$ hour to six hours after transferring to 0.9 per cent. saline.

Respiration was studied in open-type Warburg manometers with vessels of about 5 ml. capacity, containing 0.9 per cent. saline medium at 38°C. Approximately equal numbers of male and female nematode parasites were used and fluid volumes were adjusted to allow for the volume of the parasites.

Q_{O_2} values, i.e. oxygen uptake in μ l/mg. dry weight of tissue/hour, were determined in air or, when specified, in oxygen. Oxygen consumption and carbon dioxide production were determined by the direct method of Warburg (1926). Anaerobic carbon dioxide production was examined in an atmosphere of nitrogen which had been passed over copper turnings at 400°C. to remove traces of oxygen.

Neutralized potassium cyanide, with a final concentration of 0.001M, was used in the determination of cyanide inhibition except when stated otherwise. It was shown by Riggs (1945) that the previously accepted methods (Krebs 1935; Umbreit, Burris, and Stauffer 1947) employed to prevent the loss of cyanide from the medium in Warburg vessels, due to absorption by the alkali in the centre wells, were unsatisfactory. Adjustments to compensate for the removal of cyanide were therefore made empirically.

Dry weights were determined after drying to constant weight at 100°C. The results given in the tables are averages calculated from the gas changes occurring during the first 30 minutes of the experiments.

III. RESULTS

The amount of dry matter found in the species which were studied is shown in Table 1. The average figures showing the character of the respiration of the nematode parasites are listed in Table 2.

Ostertagia circumcincta and *Haemonchus contortus* were the only two species with R.Q.'s. similar to those determined by Rogers (1948) who obtained R.Q.'s. ranging from 0.6 to 0.96 from four species of nematodes examined under similar experimental conditions.

Two experiments with *Heterakis spumosa* gave R.Q.'s. of 1.0 and 1.2. Similar figures were obtained several times when *Syphacia* sp. was examined, but on two occasions the species gave the unusually high quotient of 1.7 accompanied by a pronounced cyanide activation. In all experiments the R.Q.'s. of

both species of *Strongylus* were even higher, reaching a value of 3.3 during the first 30 minutes of the experiments. Cyanide sensitivity was examined in *Strongylus vulgaris* only, inhibitions of about 15 per cent. being obtained.

TABLE 1
DRY MATTER IN SEVERAL SPECIES OF PARASITES

Species	Per cent. Dry Matter
<i>Ostertagia circumcincta</i>	19 – 21
<i>Haemonchus contortus</i>	19 – 21
<i>Syphacia obvelata</i>	23
<i>Heterakis spumosa</i>	22
<i>Strongylus equinus</i>	15 – 16
<i>Strongylus vulgaris</i>	17 – 18
<i>Paramphistomum cervi</i>	23 – 30

The results with *Paramphistomum cervi* are summarized in Table 3. Depending on the treatment of the flukes before examination, the character of the respiration, in particular the $Q_{O_2}^{CN}$ and the R.Q., varied considerably.

Immediately after removal from the host tissues the flukes' oxygen consumption was very low, but nevertheless they produced considerable amounts of carbon dioxide; the longer these flukes were kept *in vitro* the higher their oxygen consumption rose and the R.Q. decreased finally to 1.7.

TABLE 2
RESPIRATORY ACTIVITY OF NEMATODE PARASITES

Species	No. of Observations	Q_{O_2}	Q_{CO_2}	R.Q.	$Q_{CO_2}^{N_2}$	$Q_{O_2}^{CN}$	Inhibition by 0.001M KCN (%)
<i>Ostertagia circumcincta</i>	1	-7.4	5.9	0.8	3.1	-3.6	50
<i>Haemonchus contortus</i>	2	-4.6	4.0	0.87	5.3	-2.3	50 (0.02M)
<i>Syphacia obvelata</i>	3	-4.4	4.7	1.1	2.5	-2.7	40
	2	-5.0	8.6	1.7	6.7	-8.5	*
<i>Heterakis spumosa</i>	2	-3.8	4.0	1.1	2.2	-1.9	50
<i>Strongylus equinus</i>	2	-1.1	3.3	3.0			
<i>Strongylus vulgaris</i>	2	-1.1	3.6	3.3		-0.92	15

* In this experiment cyanide caused an activation of 70 per cent.

Paramphistomum cervi has a low area/volume ratio. Hence it was considered that the high R.Q. might have resulted from poor oxygen penetration and these parasites were, therefore, re-examined in an atmosphere of oxygen. The Q_{O_2} was greatly increased under these conditions and an R.Q. of 1.7 was obtained, which was similar to that found in experiments with *Syphacia*.

The effect of cyanide on the respiration of the flukes was unusual. It caused an activation of the oxygen consumption in fresh flukes of, usually,

over 100 per cent. which decreased somewhat during the period of the experiment. The activation of oxygen consumption by cyanide decreased as the time the flukes were kept *in vitro*, before the experiment, was increased (see Table 3). Carbon dioxide production was also increased by cyanide so that the R.Q. of a given experiment was unaffected by the addition of cyanide. In an atmosphere of oxygen, cyanide either inhibited up to 30 per cent. or caused only a very slight activation.

TABLE 3
RESPIRATORY ACTIVITY OF *PARAMPHISTOMUM CERVİ*

Treatment of Trematodes Before Examination	Q_{O_2}	Q_{CO_2}	R.Q.	$Q_{CO_2}^{N_2}$	Effect of 0.001M KCN
Examination in air immediately after the death of the host	- 0.03	0.7	8 and over	0.7	Over 100% activation
Examined in oxygen immediately after the death of the host	- 0.71	1.2	1.7	-	0.30% activation
Left 6 hours on host tissue at 20°C.	- 0.17	0.87	5.6	0.7	30% activation
Left 6 hours in saline at 20°C.	- 0.23	0.55	2.4	0.28	Slight inhibition
Left 6 hours in saline at 38°C.	- 0.18	0.3	1.7	0.28	No effect

IV. DISCUSSION

Certain features of the techniques used require brief comment. Thus, as the unusual action of cyanide on *Paramphistomum cervi* may suggest that the technique was defective, it should be emphasized that the same procedures were used, without unusual results, when other helminths were examined. Laser (1942) has pointed out certain limitations of the direct method of Warburg in determining respiratory quotients which apply to the present work. Anaerobic carbon dioxide determinations were not carried out in an attempt to measure anaerobic glycolysis. As nematode parasites probably produce only small amounts of lactic acid (Rogers and Lazarus 1948) but may produce considerable amounts of other acids, classical methods of determining anaerobic glycolysis may be misleading when used with these organisms. Therefore anaerobic carbon dioxide determinations were used in this investigation to give rough indications of the relative activity of anaerobic catabolic mechanisms in the species examined. However, it can be seen from the results given in Table 2 that no clear distinctions could be drawn between the different parasites examined by this method.

The oxygen consumption of all the nematodes examined was found to be greater than that of *Ascaris lumbricoides* (Laser 1944) but similar to that of the parasites examined by Rogers (1948). The trematode, *Paramphistomum cervi*, had a much lower Q_{O_2} than the nematode parasites.

The organisms examined, with the exception of *Paramphistomum cervi*, were of a similar shape but differed in size. The surface area of a given weight of tissue, therefore, varied also, and respiratory activity might best be

examined per unit surface area rather than on a weight basis. Though the dry matter in the parasites varied slightly (see Table 1) the specific gravity of the parasite tissues has been taken as constant for the purpose of estimating the relative surface area. On this assumption the relative surface areas of the nematodes used was calculated. The diameter of each species was obtained by measuring three male and three female nematodes at several points along their length and the mean average was used in calculating surface area. The relative surface area for a given weight of parasite tissue and the relative oxygen consumptions per unit surface of each parasite are shown in Table 4. Though the distinction between the respiratory activity of the trematode *P. cervi* and the several nematodes is still evident, the oxygen consumption rates of the nematode parasites show a smaller range when calculated on a surface-area basis.

TABLE 4
RELATIVE OXYGEN UPTAKE PER UNIT SURFACE AREA OF THE PARASITES

Species	Relative Surface Area	Relative Oxygen Uptake	Oxygen Uptake per Unit Surface Area
<i>Paramphistomum cervi</i>	1	0.03	0.03
<i>Strongylus equinus</i>	2	1.1	0.5
<i>Strongylus vulgaris</i>	3	1.1	0.4
<i>Haemonchus contortus</i>	12	4.6	0.4
<i>Heterakis spumosa</i>	13	3.8	0.3
<i>Ostertagia circumcincta</i>	14	7.4	0.5
<i>Syphacia obvelata</i>	18	4.7	0.3

Among the parasites examined, *Haemonchus contortus*, *Ostertagia circumcincta*, and *Paramphistomum cervi* contain haemoglobin (Rogers 1949 and unpublished data) and such organisms might, therefore, be expected to give higher Q_{O_2} 's than those which lack haemoglobin. That no such distinction was found is in agreement with the observation that haemoglobin in nematode parasites does not function as an effective oxygen carrier (Rogers 1943b).

As the R.Q. results from a number of different simultaneous reactions and does not simply indicate the type of substrate metabolized, the value of such determinations might be doubted. However, one of the possible interpretations of the high R.Q. values obtained for *Syphacia*, *Strongylus*, and *Paramphistomum* is that active carbohydrate-to-fat conversions occur in the tissues of these parasites. It is perhaps significant that the organisms giving high R.Q. values came from those regions of the host where conditions were most suitable for bacterial fermentation, e.g. the caecum of the horse and rodent and the reticulum of the ruminant. Such organisms might be expected to show considerable variation in the R.Q. as the varying degrees of interconversion occurred. This variation in the R.Q. was found for *Syphacia* and *Paramphistomum*. A change from fermentative metabolism towards a more aerobic metabolism occurred in *Paramphistomum* as the period increased during which it was incubated under aerobic conditions (see Table 3).

Cyanide was an effective inhibitor of oxygen consumption in all species of parasites which had an R.Q. below about 1.1. Higher R.Q.'s. were associated with a lower sensitivity to cyanide until, in *Paramphistomum* and, on occasions, in *Syphacia*, cyanide caused a pronounced increase in the Q_{O_2} . Activation by cyanide was not obtained when *Paramphistomum brei* was examined in the presence of methylene blue. The activation of oxygen uptake by cyanide is not commonly found when animal tissues are examined. However, von Brand and Tobie (1948) have reported that oxygen uptake of trypanosomes is stimulated by cyanide, which also causes an increase in glucose utilization.

In general, the present work has shown that the pattern of respiratory metabolism in nematode parasites may vary greatly, even among morphologically related forms such as the strongylids *Haemonchus*, *Ostertagia*, and *Strongylus*. Indeed, the nature of the respiratory activity of *Strongylus* appears more like that of the oxyurids *Syphacia* and *Heterakis* than that of *Haemonchus* and *Ostertagia*. The respiratory metabolism of the one trematode examined, *Paramphistomum cervi*, was quite different from that of the nematodes.

V. ACKNOWLEDGMENT

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THE INTERMEDIARY METABOLISM OF NEMATODE PARASITES

I. THE GENERAL REACTIONS OF THE TRICARBOXYLIC ACID CYCLE

By V. MASSEY^{*}† and W. P. ROGERS^{*}

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Summary

The oxygen consumption of brei or mince prepared from *Nematodirus filicollis*, *N. spathiger*, *Ascaridia galli*, and *Neoaplectana glaseri* was stimulated by the addition of pyruvate, α -ketoglutarate, succinate, fumarate, malate, and oxaloacetate. Citrate did not stimulate respiration. Malonate, arsenate, arsenite, pyrophosphate, and azide inhibited respiration. When brei from *Nematodirus* spp. and *Ascaridia galli* were fortified with coenzymes, the inhibition due to malonate was decreased by adding intermediates of the tricarboxylic acid cycle, and the accumulation of succinate in the malonate-poisoned brei was increased when fumarate, fumarate plus pyruvate, or citrate was added.

When calcium ions were present in the medium in which the brei was suspended, the results indicated that only isolated reactions of the tricarboxylic acid cycle were taking place because succinate failed to decrease malonate inhibition and did not accumulate even when fumarate or pyruvate was added. The characteristic reactions of the whole cycle could be obtained by omitting calcium chloride from the medium or by adding adenosinetriphosphate, diphosphopyridine nucleotide, and triphosphopyridine nucleotide.

On the basis of these results, it is suggested that some form of the tricarboxylic acid cycle functions in the tissues of the parasites.

I. INTRODUCTION

The nature of the processes in the metabolism of nematode parasites by which energy is obtained has been examined by a number of workers and generally it has been agreed that glycogen is the chief energy reserve (for references see von Brand and Jahn 1940). The anaerobic breakdown of glycogen, through hexose phosphates and triose phosphates to lactate, follows a course which is, superficially at least, similar to that in yeast and mammalian muscle and leads to the accumulation of energy in the labile phosphate bonds of adenosinetriphosphate (Rogers and Lazarus 1949). This route probably provides only a part of the anaerobic energy available to the parasites, because von Brand (1934), in a series of careful analyses, showed that the disappearance of glycogen in *Ascaris lumbricoides*, maintained *in vitro* under anaerobic conditions, was coupled with the production of lower fatty acids, chiefly valeric acid. A number of different routes have been suggested for the formation of

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valeric acid from glycogen in the tissues of nematode parasites (for references see von Brand and Jahn 1940) but there is no experimental evidence to support these hypotheses.

Whether the energy from anaerobic sources is supplemented by that obtained from oxidative mechanisms *in vivo* is not clear. There is no doubt that nematode parasites can utilize oxygen when it is available to them *in vitro* (Laser 1944; Rogers 1948); whether oxygen is always available to parasites of the alimentary tract *in vivo* is uncertain, though it does appear that conditions in the small intestine of some animals are such as to allow certain small parasites to respire quite actively (Rogers 1949*a*, 1949*b*). Some workers (Harnish 1933, 1935; Krüger 1937) are of the opinion that, whatever aerobic mechanisms exist in *Ascaris lumbricoides*, they cannot be regarded as processes which yield energy, or that the energy so formed could not be used. However, Slater (1925) has shown that *Ascaris lumbricoides*, when continuously stimulated to activity *in vitro*, lives longer if oxygen is available; and von Brand (1937), using the same parasite, found that after a period of anaerobiosis, glycogen resynthesis occurs when oxygen becomes available. These results suggest that oxidative mechanisms can be used by these parasites to provide useful energy.

Very little is known of the routes of aerobic catabolism in the tissues of nematode parasites. The amounts of glycogen used and the amounts of valeric acid and carbon dioxide produced by *Ascaris lumbricoides* under aerobic and anaerobic conditions were compared by von Brand (1934). Less glycogen was used when oxygen was available to the parasites but the results indicated that the oxidation of carbohydrates was partial and that probably only a small proportion was completely oxidized to carbon dioxide and water. Harnish (1935) has suggested that the aerobic route might involve the oxidation of isovaleric acid to acetoacetic acid, or to β -hydroxybutyric acid, with subsequent formation of acetone and carbon dioxide. Without denying the value of previous work and the hypotheses that have been formulated, it seems that the rational approach to the determination of oxidative routes in the tissues of nematode parasites is first to seek information on the importance of accepted sources of energy such as the tricarboxylic acid cycle (Krebs 1943). Though the respiratory quotients of large parasites respiring in air-saturated saline are high, the quotients of smaller parasites (Rogers 1948; Lazarus 1950) are not incompatible with the suggestion that some carbohydrate oxidation takes place through a mechanism of the nature of the tricarboxylic acid cycle.

This paper describes experiments designed to show whether cyclic oxidations similar to those of the tricarboxylic acid cycle occur in the tissues of three different nematode parasites. The results of a detailed examination of specific reactions of the cycle will be described in a later publication.

II. BIOLOGICAL MATERIALS

Neoaplectana glaseri, which is naturally a parasite of the Japanese beetle, was grown *in vitro* on sterile liver slices supported on neutral agar. The mixed populations of males, females, and larvae were washed from the cultures with

0.5 per cent. saline, concentrated by centrifuging and washed several times before use. *Nematodirus filicollis* and *N. spathiger*, which were not separated for use, were obtained from naturally infested sheep. *Ascaridia galli* was obtained from experimentally infected chickens.

Brei was prepared by grinding the parasites in a chilled mortar with quartz sand. The machine described by Seevers and Shideman (1941) was used for mincing tissues. Pigeon breast muscle, which was used for comparison in some experiments, was prepared similarly. Krebs-Ringer phosphate, at pH 7.3, with or without calcium chloride, or 0.01M phosphate buffer, was used as a medium, 9 volumes to 1 volume of brei or mince. In some instances cofactors such as nicotinamide were ground with the tissues.

III. METHODS

Oxygen uptakes were determined at 37°C. by the direct method of Warburg (1926). Substrates, cofactors, and inhibitors were dissolved in Krebs-Ringer phosphate and neutralized before being added to the brei in the flasks. As a rule, oxygen consumption was calculated from results obtained during the first 30 minutes after equilibration. Oxygen uptake was not limited by the rate of shaking of the Warburg manometers.

Succinate was extracted from brei or mince by continuous extraction with ether (Krebs 1937) and determined manometrically with succinoxidase from pig's heart (Cohen 1940). Citric acid was determined colorimetrically by Krebs and Eggleston's (1944) modification of the method of Pucher, Sherman, and Vickery (1936). Cytochrome *c* was prepared from ox heart by the method of Keilin and Hartree (1937) and estimated spectrophotometrically; coenzymes I and II were prepared by the methods of Williamson and Green (1940) and Warburg, Christian, and Griese (1935) respectively. Adenosinetriphosphate was prepared from rabbit muscle as the barium salt, from which the sodium salt was obtained as required immediately before an experiment.

IV. PROCEDURE AND RESULTS

(a) Effects of Added Oxygen Carriers on Respiration Rates

The addition of methylene blue to brei prepared from *Ascaridia galli* or *Nematodirus* spp. up to a final concentration of M/1000 caused, at times, an increased oxygen consumption up to 25 per cent. above that in control flasks. The stimulating effect of methylene blue was more consistent and greater in the presence of added substrates such as succinate. No consistent differences were noted between the tissues of the two parasites.

The addition of cytochrome *c* to an approximate concentration of 2×10^{-5} M gave results similar to those obtained with methylene blue. In the experiments described in the following parts of this paper, methylene blue, usually at a final concentration of 0.0003M, was added as a routine measure.

(b) Effects of Added Cofactors on Respiration Rates

Cocarboxylase, riboflavin, nicotinamide, pyridoxin, diphosphopyridine nucleotide, and triphosphopyridine nucleotide had no consistent effect on the endogenous respiration of brei in Krebs-Ringer phosphate. The coenzymes, however, had a pronounced and consistent effect on oxygen uptake in the

TABLE 1
EFFECT OF COFACTORS ON SUBSTRATE-INHIBITOR RELATIONSHIPS

System	<i>Nematodirus</i> spp.		<i>Ascaridia galli</i>	
	Oxygen Uptake (μl.)	Change (%)	Oxygen Uptake (μl.)	Change (%)
No additions	92		141	
+ Malonate 0.01M	54	- 41	104	- 26
+ Succinate 0.01M	207	+ 125	236	+ 67
+ Malonate 0.01M } + succinate 0.01M }	75	- 64	129	- 45
+ α-Ketoglutarate 0.01M	131	+ 42	208	+ 47
+ α-Ketoglutarate 0.01M } + malonate 0.01M }	80	- 39	142	- 32
+ Cofactors	145	+ 58	185	+ 31
+ Cofactors } + malonate 0.01M }	62	- 57	125	- 35
+ Cofactors } + succinate 0.01M }	214	+ 47	222	+ 20
+ Cofactors } + succinate 0.01M } + malonate 0.01M }	153	- 28	213	- 5
+ Cofactors } + α-ketoglutarate 0.01M }	161	+ 11	209	+ 13
+ Cofactors } + α-ketoglutarate 0.01M } + malonate 0.01M }	98	- 39	194	- 7

The results were obtained from the first 20 minutes of the experiments. Each flask contained 1 ml. of parasite mince and methylene blue to a concentration of 0.0003M. The cofactor mixture, which was added from the side-arm of the Warburg flask after equilibration at 37°C., contained 1 mg. of coenzyme I (purity 40 per cent.), 0.1 mg. of coenzyme II (purity 15 per cent.), and 2.5 mg. of adenosinetriphosphate. The whole system was buffered with Krebs-Ringer phosphate solution at pH 7.3.

presence of malonate and added succinate. Without the addition of coenzymes, succinate added to malonate-inhibited brei failed to lift the inhibition; indeed, the inhibition was increased (Massey and Rogers 1949). When the brei or

mince was fortified with coenzymes I and II, the addition of succinate or α -ketoglutarate led to decreased malonate inhibition in the accepted manner. Typical results obtained from brei prepared from *Ascaridia galli* and *Nematodirus* spp. are shown in Table 1.

The effect of calcium ions on respiration was determined by comparing the oxygen consumption of brei prepared in Krebs-Ringer phosphate, with and without calcium chloride. Typical results are shown in Table 2. Endogenous respiration was decreased when calcium ions were present but succinate gave a greater stimulation. In the absence of calcium ions succinate was more effective in lifting malonate inhibition. Similar results were obtained from preparations of *Nematodirus* spp. and *Ascaridia galli*.

TABLE 2
EFFECT OF CALCIUM IONS ON SUCCINATE-MALONATE RELATIONSHIPS IN
NEMATODIRUS SPP.

	System	Oxygen Uptake (μ l.)	Change (%)
	No additions	165	
Medium containing calcium	+ Malonate 0.01M	71	- 57
	+ Succinate 0.01M	233	+ 41
	+ Succinate 0.01M } - malonate 0.01M }	117	- 50
	No additions	180	-
Medium without calcium	+ Malonate 0.01M	91	- 50
	+ Succinate 0.01M	208	+ 15
	+ Succinate 0.01M } + malonate 0.01M }	151	- 27

Each flask contained 1 ml. of brei, methylene blue to a final concentration of 0.0003M, and was buffered with Krebs-Ringer phosphate solution of pH 7.3, with or without calcium chloride according to the particular experiment.

(c) Effect of Added Substrates on Respiration Rates

The effects of intermediates of the tricarboxylic acid cycle on the respiration of parasite brei in Krebs-Ringer phosphate were determined during the first 30 minutes of the experiment after equilibration. The percentage stimulation arising from the addition of the substrates is shown in Table 3. Though the degree of stimulation varied greatly in different preparations, it is clear that all the metabolites added, except citrate, actively stimulated respiration. The failure of citrate to stimulate respiration is difficult to explain in view of the fact that it was rapidly metabolized (see later in this paper). The effect may be partly attributed to its action in competing with its oxidation products, succinate and malate (Krebs 1943).

The general implication of the results shown in Table 3 is that some form of the tricarboxylic acid cycle functions in the tissues of the three nematode parasites examined.

TABLE 3
EFFECT OF ADDED SUBSTRATES ON RESPIRATION

Substrate	<i>Nematodirus</i> spp.	<i>Ascaridia</i> <i>galli</i>	<i>Neoaplectana</i> <i>glaseri</i>
Pyruvate 0.01M	0-45	20-30	—
Mean	25 (6)	25 (5)	—
Citrate 0.01M	No effect (6)	—	—
α -Ketoglutarate 0.01M	0-45	20-120	—
Mean	25 (9)	60 (4)	—
Succinate 0.01M	50-140	50-135	45-110
Mean	90 (10)	100 (4)	60 (3)
Fumarate 0.01M	0-60	15-90	25-35
Mean	40 (8)	60 (6)	30 (2)
Malate 0.01M	20-35	15-70	10-80
Mean	25 (4)	40 (3)	40 (3)
Oxaloacetate 0.01M	0-50	20-25	—
Mean	15 (6)	25 (3)	—

The results refer to the percentage stimulation of respiration over the first 30 minutes of the experiments. The figures within brackets give the number of experiments from which the results were taken.

(d) Effect of Inhibitors on Respiratory Rates

Inhibitors were dissolved in Krebs-Ringer phosphate and neutralized with sodium hydroxide before being added to brei or mince prepared in Krebs-Ringer phosphate. The degree of inhibition was calculated as the percentage fall in oxygen uptake below that in the control vessels during a period of 30 minutes after equilibration. The results are shown in Table 4.

The effect of malonate on respiration varied with different lots of material, and only at higher concentrations, 0.01M and 0.02M, was inhibition consistently obtained. Pyrophosphate, arsenite, arsenate, and azide were all effective inhibitors of respiration in preparations from *Ascaridia galli* and *Nematodirus* spp.

The action of succinate in lifting malonate inhibition in brei fortified with coenzymes I and II and adenosinetriphosphate has already been described. The effect of other metabolites of the tricarboxylic acid cycle on malonate inhibition is shown by the results of typical experiments set out in Tables 5 and 6. Malate, fumarate, pyruvate and fumarate were all effective in reducing malonate inhibition in brei prepared from *Nematodirus* spp. and *Ascaridia galli*.

(e) *Formation of Succinate in Malonate-Poisoned Brei*

The succinate concentration in brei suspended in Krebs-Ringer phosphate containing 0.0003M methylene blue was determined before and after shaking for 2 hours at 37°C. Under these conditions succinate formation was negligible, even when both fumarate, 0.01M, and malonate, 0.01M, were present (Massey and Rogers 1949). However, in similar experiments carried out with pigeon breast muscle an appreciable accumulation of succinate was recorded.

TABLE 4
EFFECT OF INHIBITORS ON RESPIRATION

Inhibitor	<i>Nematodirus</i> spp.	<i>Ascaridia</i> <i>galli</i>	<i>Neoaplectana</i> <i>glaseri</i>
Malonate 0.001M	0.5	0.30	No effect (2)
Mean	1 (5)	10 (5)	
Malonate 0.01M	25.50	20.4	10.20
Mean	40 (11)	30 (7)	15 (2)
Malonate 0.02M	40.55	35.45	—
Mean	50 (4)	40 (2)	
Pyrophosphate 0.01M	40.50	15 (1)	—
Mean	45 (2)		
Arsenite 0.001M	70 (1)	50 (1)	—
Arsenite 0.01M	75 (1)	55 (1)	—
Arsenate 0.01M	30 (1)	30 (1)	—
Azide 0.01M	50 (1)	45 (1)	—

The figures given refer to the percentage inhibition of respiration during the first 30 minutes of the experiments. The figures within brackets give the number of experiments from which the results were taken.

The addition of coenzymes I and II and of adenosinetriphosphate to brei prepared from *Nematodirus* spp. had a pronounced effect on the accumulation of succinate, which was appreciable even in the absence of malonate. Succinate accumulation in brei from *Ascaridia galli* could also be shown when the brei was fortified with the nucleotides. Typical results are shown in Table 7. Succinate accumulation in pigeon breast muscle, examined under the same conditions as the preparations from the parasites, is shown in the same table for comparison.

(f) *Utilization of Citrate by the Tissues of the Parasites*

The utilization of citrate in the presence and absence of malonate with the subsequent formation of succinate was examined in brei prepared in Krebs-Ringer phosphate. The concentrations of citrate and succinate were determined immediately after adding the substrates to the brei and after the mixtures

had been shaken for 2 hours at 37°C. The results of a typical experiment are shown in Table 8; the percentage conversion of succinate equivalents of citrate to succinate have been calculated. Under the conditions of these experiments, when coenzymes were not added, the accumulation of succinate in the absence

TABLE 5
EFFECT OF SUBSTRATES ON MALONATE INHIBITION IN *NEMATODIRUS* SPP.

System	Oxygen Uptake (μ l.)	Inhibition Due to Malonate (%)
No additions	155	
+ Malonate 0.005M	131	16
+ Fumarate 0.01M } + pyruvate 0.01M }	197	
+ Fumarate 0.01M } + pyruvate 0.01M } + malonate 0.005M }	194	2
No additions	102	
+ Malonate 0.01M	63	39
+ Fumarate 0.01M	139	
+ Fumarate 0.01M } + malonate 0.01M }	137	2
No additions	190	
+ Malonate 0.02M	113	40
+ Malate 0.01M	242	
+ Malate 0.01M } + Malonate 0.02M }	196	20

Each flask contained 1 ml. of parasite brei and methylene blue to a final concentration of 0.0003M, and was buffered with Krebs-Ringer phosphate solution pH 7.3. The results given above were obtained from the first 30 minutes of the experiments.

of citrate was negligible even when malonate was present (Massey and Rogers 1949). In spite of the fact that citrate failed to stimulate respiration in brei it would appear that it was utilized with the formation of α -ketoglutarate and succinate. The interpretation of the results of these experiments is complicated by the fact that citrate may inhibit the further oxidation of its primary oxidation product, α -ketoglutaric acid, and because malonate may increase the utilization of added citrate (Stone, Lipton, and Goldinger 1941). In the absence of malonate, citrate competes with its oxidation products; malonate increases the

utilization of citrate by reducing this competition (Krebs 1943). It should be pointed out that the method used for the estimation of succinate really determines succinate plus α -ketoglutarate.

TABLE 6
EFFECT OF SUBSTRATES ON MALONATE INHIBITION IN *ASCARIDIA GALLI*

System	Oxygen Uptake (μ l.)	Inhibition Due to Malonate (%)
No additions	153	
+ Malonate 0.02M	100	35
+ Fumarate 0.01M	204	
+ Fumarate 0.01M } + malonate 0.02M }	198	3
No additions	96	
+ Malonate 0.001M	71	26
+ Pyruvate 0.01M } + fumarate 0.01M }	143	
+ Pyruvate 0.01M } + fumarate 0.01M } + malonate 0.001M }	150	No inhibition
No additions	96	
+ Malonate 0.01M	73	24
+ Malate 0.01M	110	
+ Malate 0.01M } + malonate 0.01M }	102	7

Each flask contained 1 ml. of parasite brei and methylene blue to a final concentration of 0.003M, and was buffered with Krebs-Ringer phosphate solution pH 7.3. The results given above were obtained from the first 30 minutes of the experiments.

V. DISCUSSION

It is probable that the action of calcium ions on the respiration of the tissue preparations of the parasites was due to the activation of phosphatases (Dubois and Potter 1943) which led to the rapid destruction of diphosphopyridine nucleotide, triphosphopyridine nucleotide, and adenosinetriphosphate. This action could be overcome by omitting calcium chloride from the medium or by the addition of relatively large amounts of the coenzymes. The suggestion that the action of calcium ions was due to the activation of phosphatases

is supported by the results obtained with brei which was prepared by grinding the parasites with a little phenothiazine. Phenothiazine is an inhibitor of phosphatases (Lazarus, unpublished data), and phenothiazine-poisoned brei gave results similar to those obtained when calcium chloride was omitted from the medium (see Table 9).

TABLE 7
EFFECT OF MALONATE ON SUCCINATE FORMATION

System	<i>Nematodirus</i> spp.	<i>Ascaridia</i> <i>galli</i>	Pigeon Breast Muscle
Fumarate 0.01M } + pyruvate 0.01M }	0.30	0.34	0.28
Fumarate 0.01M } + pyruvate 0.01M } + malonate 0.01M }	0.30	0.36	1.33
Fumarate 0.01M } + pyruvate 0.01M } + cofactors }	0.40	0.51	—
Fumarate 0.01M } + pyruvate 0.01M } + cofactors } + malonate 0.01M }	1.25	1.03	—

The figures represent milligrams of succinate which accumulated during shaking at 37°C. for 2 hours. Each flask contained 10 ml. of brei and methylene blue to a final concentration of 0.0005M. Each flask was buffered with Krebs-Ringer phosphate solution without calcium chloride of pH 7.3. The cofactor mixture which was added contained 10 mg. coenzyme I (purity 40 per cent.), 0.2 mg. coenzyme II (purity 15 per cent.), and 10 mg. adenosinetriphosphate.

TABLE 8
EFFECT OF MALONATE ON CITRATE UTILIZATION IN *NEMATODIRUS* SPP.

System	Initial Citrate	Citrate after 2 Hours	Initial Succinate	Succinate after 2 Hours	Conversion of Citrate to Succinate
+ Citrate	2.5 mg.	1.3 mg.	0.20 mg.	0.51 mg.	42%
+ Citrate } + malonate 0.02M }	2.5 mg.	2.0 mg.	0.20 mg.	0.42 mg.	71%

Each flask contained 5 ml. of parasite brei, citrate to a final concentration of 1 mg. per ml., methylene blue to a final concentration of 0.0002M, and was buffered with Krebs-Ringer phosphate solution pH 7.3. Samples for analysis were taken immediately after the addition of brei, and after 2 hours' shaking at 37°C.

When the coenzyme concentration of the brei was very low, oxidative activity would be restricted to isolated oxidations of individual intermediates of the tricarboxylic acid cycle; coenzyme-linked reactions would be weak and

the cycle would not function as a whole. Under such circumstances the failure of succinate to accumulate in malonate-poisoned brei to which fumarate and pyruvate had been added would be expected.

TABLE 9
EFFECT OF PHENOTHIAZINE ON MALONATE-SUCCINATE RELATIONSHIPS

System		<i>Nematodirus</i> spp.		<i>Ascaridia</i> <i>galli</i>	
		Oxygen Uptake (μ l.)	Change (%)	Oxygen Uptake (μ l.)	Change (%)
Brei without pheno- thiazine	No additions	109		43	
	+ Malonate 0.01M	61	- 44	33	- 23
	+ Succinate 0.01M	200	+ 83	83	+ 93
	+ Malonate 0.01M } + succinate 0.01M }	101	- 49	50	- 44
Brei ground up with pheno- thiazine	No additions	85		60	
	+ Malonate 0.01M	58	- 32	49	- 18
	+ Succinate 0.01M	91	+ 7	76	+ 27
	+ Malonate 0.01M } + succinate 0.01M }	85	- 7	78	+ 3

Each flask contained 1 ml. of brei, methylene blue to a final concentration of 0.0003M, and was buffered with Krebs-Ringer phosphate solution of pH 7.3. The results given above were obtained from the first 20 minutes of the experiments.

When conditions were such that an adequate concentration of coenzymes was maintained in the parasite brei, several characteristic reactions of the tricarboxylic acid cycle were demonstrated:

- (1) The addition of fumarate, malate, oxaloacetate, pyruvate, α -ketoglutarate, or succinate, all of which are intermediates in the cycle, caused an increase in respiration.
- (2) In the presence of malonate, succinate accumulated in respiring brei, and the amount formed was increased when fumarate or pyruvate was added.
- (3) Malonate inhibited respiration and this inhibition was lessened when intermediates of the tricarboxylic acid cycle were added.
- (4) Added citrate was utilized by the parasite brei, and utilization was inhibited by malonate. In the presence of citrate, succinate accumulation was increased, not only because citrate or its products inhibited succinic dehydrogenase, but also because citrate was apparently converted to succinate.

- (5) It has been shown (Massey and Rogers, unpublished data) that citrate was formed in parasite brei as a result of the condensation of oxaloacetate and acetate or its products.
- (6) The results obtained with inhibitors were largely in accordance with the view that the tricarboxylic acid cycle was functioning in the parasite brei. Thus pyrophosphate, which is considered to be an inhibitor of succinic dehydrogenase (Leloir and Dixon 1937) though it does not cause an accumulation of succinate (Krebs 1943), and arsenite, which inhibits the oxidative decarboxylation of α -ketoglutarate (Krebs and Johnson 1937), were effective inhibitors of the respiration of the brei. Malonate, however, was often a poor inhibitor when used in low concentrations. Though azide and arsenate chiefly inhibit systems outside the tricarboxylic acid cycle, their action would generally lead to lessened activity within the cycle.

Some of the results obtained in the present investigation are not completely in accord with the hypothesis that the tricarboxylic acid cycle was functioning in the parasite brei. Thus, though citrate was actively utilized by the brei, on no occasion did it cause an increase in respiration and it is difficult to agree that this was due to the fact that the inhibition of succinic dehydrogenase was always such that it exactly negated the stimulation produced by the increase in the concentration of substrates. Again, malonate was a relatively poor inhibitor of the respiration of parasite brei. In low concentrations, 0.004M, malonate is a specific inhibitor of succinic dehydrogenase; at higher concentrations, 0.02M, magnesium-activated enzymes of the tricarboxylic acid cycle are also inhibited (Pardee and Potter 1949). The results obtained when the tissues of the parasites were examined suggests that either the succinic dehydrogenase was relatively insensitive to malonate, or that the dehydrogenase was relatively less important in the respiratory mechanisms of the parasite than in mammalian tissue, or that some component in the parasite brei lessened the efficiency of malonate as an inhibitor. However, in view of the positive evidence already given, which indicated that the tricarboxylic acid cycle was functioning in the parasite brei, these inconsistencies are not serious.

The tricarboxylic acid cycle has been shown to function in the tissues of a wide variety of mammals. It also functions in certain protozoa (Evans 1946) and insects (Barron and Tahmisian 1948). The precise details of the cycle, however, are not known and it would be unprofitable to discuss the apparent differences in the cycle as it occurs in the tissues of different animals.

The parasites used in this investigation probably differed greatly in the nature of their metabolism *in vivo*. *Neoplectana glaseri* is an obligate aerobe when cultured *in vitro* in a medium which is adequate for continued growth and reproduction (Massey and Rogers, unpublished data) and is probably essentially aerobic *in vivo* also. Though some oxygen is present in the contents of the small intestine of sheep, close to the mucosa, *Nematodirus* spp. are probably partially anaerobic *in vivo*, and the large *Ascaridia galli* in the small intestine of chickens, is probably largely anaerobic (Rogers 1949a, 1949b).

Yet it would appear from the results given in this paper that all these forms possess mechanisms whereby carbohydrate is oxidized. And under certain circumstances at least, the parasites can usefully utilize the energy from oxidative mechanisms. Thus, when *Ascaridia galli* and *Nematodirus* spp. are maintained *in vitro* in media containing sufficient penicillin and streptomycin to limit bacterial growth, egg production is greatly reduced when oxygen is excluded from the medium though the parasites live for a considerable period (Rogers, unpublished data). It would appear that the metabolic routes which can function in *Ascaridia galli* and *Nematodirus* spp. may be very similar, but under the conditions prevailing *in vivo*, the particular mechanisms used to obtain energy may vary considerably.

Neoplectana glaseri is similar, from many points of view, to free-living nematodes, and it would appear that adaptation to parasitism of the alimentary tract may involve the development of mechanisms for the anaerobic catabolism of pyruvate.

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AN ELECTRON MICROSCOPIC INVESTIGATION OF THE CELL WALL ORGANIZATION OF CONIFER TRACHEIDS AND CONIFER CAMBIUM

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Summary

An electron micrograph of a replica of the inner surface of the secondary cell wall of a conifer tracheid, demonstrating the almost transverse orientation of the microfibrils in this layer, is presented. This evidence provides confirmation of the type of cell wall organization of conifer tracheids proposed in other investigations on the basis of X-ray and optical evidence and of microscopic examination. The existence of fibrils of 50-100 Å in diameter has been demonstrated in cell wall fragments obtained by the disintegration of cambium initials and of conifer tracheids. It is suggested that these microfibrils may correspond to the "micelles" or "crystalline regions" inferred from X-ray examination.

I. INTRODUCTION

Structural investigations of the cell walls of plants include the study of both the nature of the structural units of which they are composed and of the way in which these structural units are arranged with respect to the morphological axes of the cells (i.e. a study of the cell wall organization). The nature of the structural units ("micelles" or "crystalline regions") of the cell wall has been summarized recently by Hermans (1949) as well as by Frey-Wyssling (1936) and Preston (1939). In the two latter publications the cell wall organization of a number of cell types is also described.

In the past, indirect methods using X-ray diffraction and polarization optics have been employed in these investigations. From the use of these techniques it was concluded that the "micelles" of the cell wall are at least 600 Å in length and 50-70 Å in their lateral dimensions (Hengstenberg and Mark 1928). With the development of the electron microscope, however, a direct approach is possible since the dimensions of the "micelles" as deduced from X-ray examinations lie within the range of particle size which can be resolved by this instrument.

Details of cell wall organization have already been published by Preston *et al.* (1948), Frey-Wyssling, Muhlethaler, and Wyckoff (1948), and Muhlethaler (1949), and the nature of the crystalline regions referred to by Svedberg (1949). In the present paper, of which a preliminary account has been published (Hodge and Wardrop 1950), both these aspects of cell wall studies are discussed with reference to the cell wall of conifer tracheids, and of conifer cambium.

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II. EXPERIMENTAL

(i) *Material*.—Wood from the eighteenth and twentieth annual rings of a specimen of *Pseudotsuga taxifolia* (Lamb.) Britt. was examined. The cell wall organization of the cambium of this species was also studied. The cambium was collected in the manner described by Priestley, Scott, and Malins (1933).

(ii) *Preparation of Material for Study by Replica Methods*.—Replica methods were employed to study the cell wall organization of conifer tracheids since the orientation of the specimen can be easily determined during examination. Thin ($10\ \mu$) tangential longitudinal sections were cut after softening in boiling water. The sections were placed on a glass slide, shadowed with gold and covered with a thin film of "Formvar." They were then removed from the gold-"Formvar" film, so that a surface replica of the specimen was obtained. A micrograph (reverse print) obtained in this way is shown in Plate 2. The spiral thickenings of the cell wall, as shown in Plate 1, adhered to the gold-"Formvar" film as it was separated from the specimen so that they appear in the electron micrograph as white diagonal bands, between which the true replica of the inner surface of the cell wall can be seen.

(iii) *Preparation of Material for Direct Examination*.—For studying the nature of the crystalline regions, direct examination was found to be more satisfactory than the replica method. For this purpose wood in the form of $30\ \mu$ sections was disintegrated in a Waring Blendor (Muhlethaler 1949). A drop of the suspension of the disintegrated cell walls was then mounted on a collodion film supported by a wire specimen mount, shadowed with uranium, and examined directly in the electron microscope.

Specimens examined in this way were:

- (i) untreated wood,
- (ii) wood delignified by treatment with hydrogen peroxide and acetic acid,
- (iii) wood delignified by treatment with sodium hydroxide and chlorine water (Cohen and Dadswell 1939), and
- (iv) cambium, after a preliminary extraction with 0.1N hydrochloric acid to remove pectins, etc.

Electron micrographs of these specimens are shown in Plates 3-10.

III. DISCUSSION

In Plate 2, the white diagonal bands correspond to the spiral cell wall thickenings visible by ordinary microscopic examination (Plate 1), so that the micrograph is a replica of the inner surface of the cell wall. In the specimens examined the spiral thickenings were inclined at 60 degrees to the longitudinal tracheid axis. Between the thickenings, a set of striations, inclined at 80 degrees to the longitudinal tracheid axis, is visible.

From optical evidence (Kerr and Bailey 1934; Wardrop and Preston 1947) it was concluded that the secondary cell wall of conifer tracheids consists of three layers in which the crystalline regions are oriented to form a flat spiral in the outer and inner layers, and in the central layer a steep spiral with respect to the longitudinal tracheid axis. In Plate 2, the presence of the spiral thickenings makes it clear that the striations visible are present in the inner layer of the secondary wall. Assuming that these striations correspond in direction to that of the micelles, then the direction of the striations corresponds to the orientation expected on the basis of the optical evidence. Since the presence of a steep spiral in the central layer has been demonstrated by optical (Wardrop and Preston 1947) and X-ray evidence (Preston 1946), the demonstration of a flat spiral in the inner layer confirms the type of organization of the cell wall of conifer tracheids originally proposed by Kerr and Bailey (1934).

Specimens examined directly after disintegration in a Waring Blendor were not as suitable as those examined by the replica technique for determination of cell wall organization since the orientation of the morphological axes of the specimens was unknown. However, specimens of this kind were more suitable for study of the crystalline regions.

Part of the cell wall of cambium is shown in Plate 3. In contrast to Plate 2, distinct microfibrils about 50-100 Å in diameter are visible. A tendency towards a direction of preferred orientation is apparent (top left to bottom right in the micrograph). The poor orientation of the microfibrils is in agreement with the considerable dispersion of the crystalline regions indicated by optical and X-ray studies of cambium (Preston and Wardrop 1949).

In the micrographs of the wood macerated with hydrogen peroxide and acetic acid three types of organization are apparent (Plates 4, 5, and 6). In Plate 4, the absence of any preferred orientation of the microfibrils present and its similarity to Plate 3 suggest that the structure shown may be that in the primary cell wall, detached from the tracheids. In Plate 5, the microfibrils possess a direction of preferred orientation but are aggregated to give a reticulate appearance. In contrast to this the microfibrils in Plate 6 are perfectly aligned with respect to one another. Those specimens which were delignified, using the method described by Cohen and Dadswell (1939), before disintegration show an apparently crossed fibrillar type of structure (Plate 7) in which the microfibrils intersect at about 70 degrees. This type of structure was not observed in specimens delignified with hydrogen peroxide and acetic acid.

At present it is difficult to assign the structures seen in Plates 5, 6, and 7 to their correct cell wall layers. In attempting this, the known properties of the several layers of the secondary cell wall are of assistance, assuming that the orientation of the microfibrils in the photographs is that of the crystalline regions of cellulose observed by X-ray methods. Thus in Plate 6, the perfect parallel alignment of the microfibrils suggests that this type of organization is that of the central layer of the secondary wall, in which optical and X-ray

evidence suggest there is little dispersion of the crystalline regions about the direction of preferred orientation (Preston 1946; Wardrop and Preston 1947).

The more reticulate structure of the specimen shown in Plate 5 would be more consistent with the properties of the outer layer of the secondary wall in which considerable dispersion of the crystalline regions about the direction of orientation is known to exist (Wardrop and Preston 1947) and which is lower in cellulose content than the central layer (Kerr and Bailey 1934; Lange, personal communication 1948). The crossed fibrillar structure observed in Plate 7 could then be regarded as a specimen in which either the outer or inner layer is superimposed upon the central layer, and Plate 5 represents the outer layer separated from it. An alternative interpretation would be that Plate 7 represents the outer layer which possesses a crossed fibrillar structure of its own and Plate 5 represents one of these layers which has been separated by the more drastic maceration technique used in preparing this specimen. The optical properties would preclude the latter alternative being applicable to the central layer. However, it is hoped that these points will be resolved during further investigations now in progress.

Evidence supporting the conception that lignin is located between the crystalline regions (Astbury, Preston, and Norman 1935; Preston and Allsopp 1939) can be seen in Plates 6 and 9. In the latter electron micrograph, which shows a fragment of untreated wood, the microfibrils give the impression of being embedded in a matrix, while they are visible with considerably greater definition in Plate 6 in which the specimen was pretreated to remove lignin.

In Plate 8, the units are less than 100 Å and more than 50 Å in diameter. In view of X-ray evidence which indicates the crystalline regions to be 50-70 Å in diameter it is apparent that these units may well be the micelles of early workers. The lack of any definite length of these units and their tendency to anastomose freely suggest that the concept proposed by Meyer (1942) may most nearly correspond to the structure actually present. The isolation of particles about 50 Å in diameter and 500 Å in length after acid hydrolysis reported by Svedberg (1949) might be attributed to the existence of weak points or discontinuities in the crystal lattice about 500 Å apart. However, there is no visual evidence in the present electron micrographs of regions susceptible to chemical attack. It may be pointed out that such chemical susceptibility may arise either from the presence of "amorphous regions" as postulated in the "fringed micelle" hypothesis, or to crystal lattice distortions as contained in the Meyer concept of the crystalline regions. A further feature of Plate 8 is that the microfibrils appear to be composed of a linear arrangement of small particles. This behaviour was first observed by Kinsinger and Hock (1946) using chromium and gold shadowing and lead acetate staining techniques and independently by Farrant (1947) using gold and platinum shadowing.

It remains to consider the relation of the ultimate microfibrils or crystalline regions (micelles) observed during the above investigations to the microscopic structures isolated from, or observed in, cell walls by various workers



Spiral thickenings of the cell wall in tracheids of *Pseudotsuga taxifolia*. Light micrograph $\times 400$.





"Formvar" replica of gold-shadowed inner surface of a tracheid of *Pseudotsuga taxifolia*. The white diagonal bands are spiral thickenings of the cell wall adhering to the replica similar to those in Plate 1, and between them can be seen the inner layer of the secondary cell wall with prominent striations.





Pseudotsuga taxifolia. Portion of the cambium disintegrated in a Waring Blendor.
Uranium-shadowed.





Pseudotsuga taxifolia. This is probably portion of the primary wall detached from delignified tracheids during disintegration in a Waring Blendor. Uranium-shadowed.



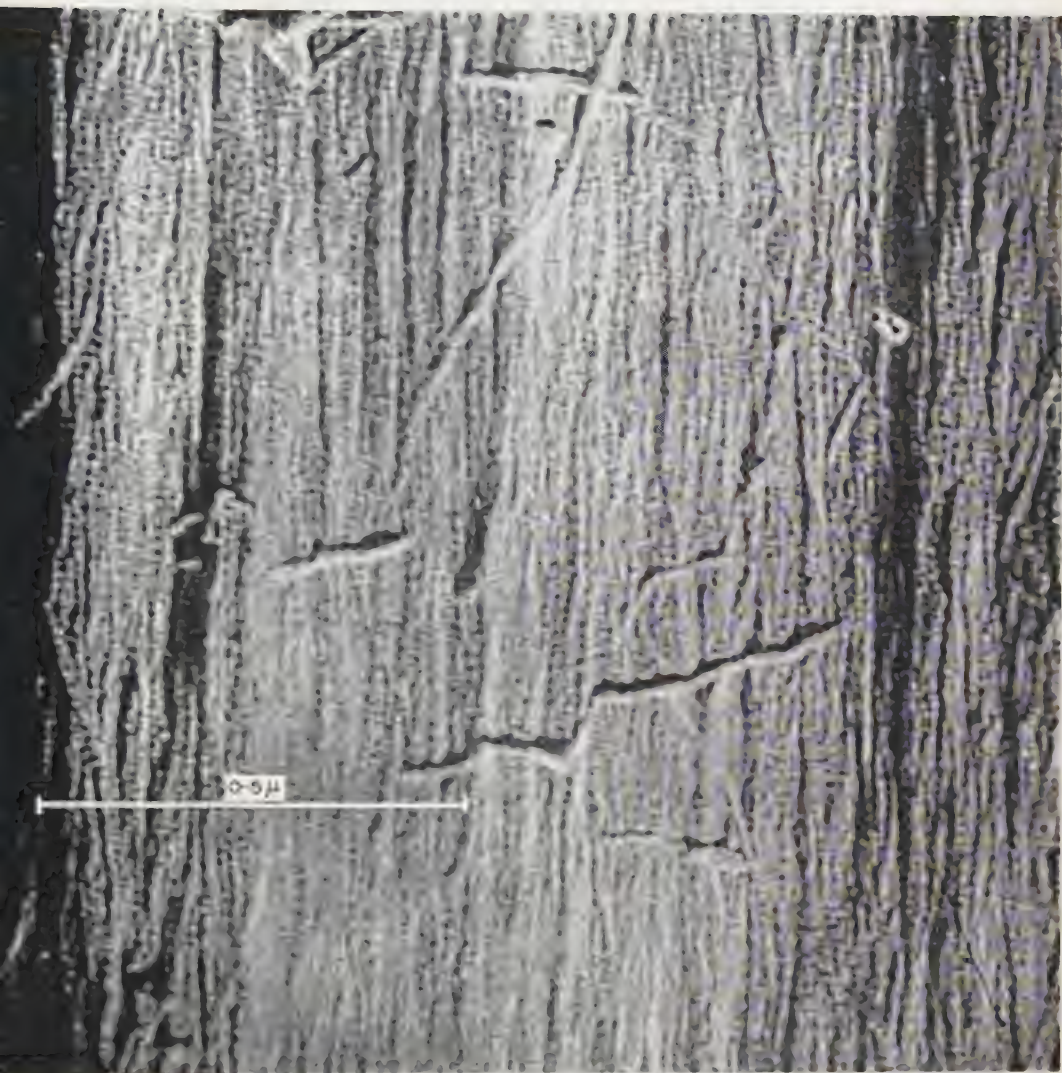
Pseudotsuga taxifolia. Fragment of the secondary cell wall of delignified tracheids, disintegrated in a Waring Blendor and uranium-shadowed.



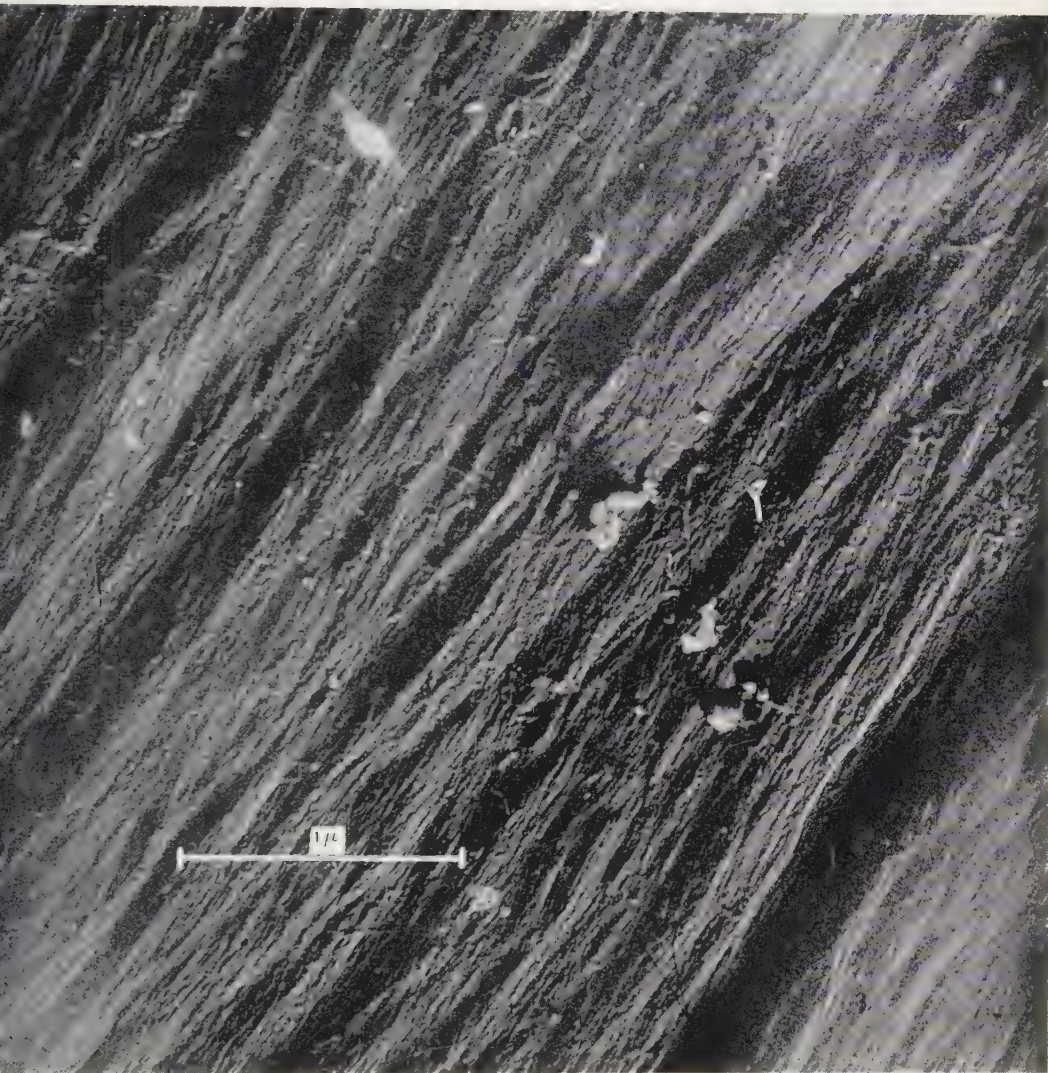
Pseudotsuga taxifolia. Fragment of the secondary cell wall of delignified tracheids, disintegrated in a Waring Blendor and uranium-shadowed.



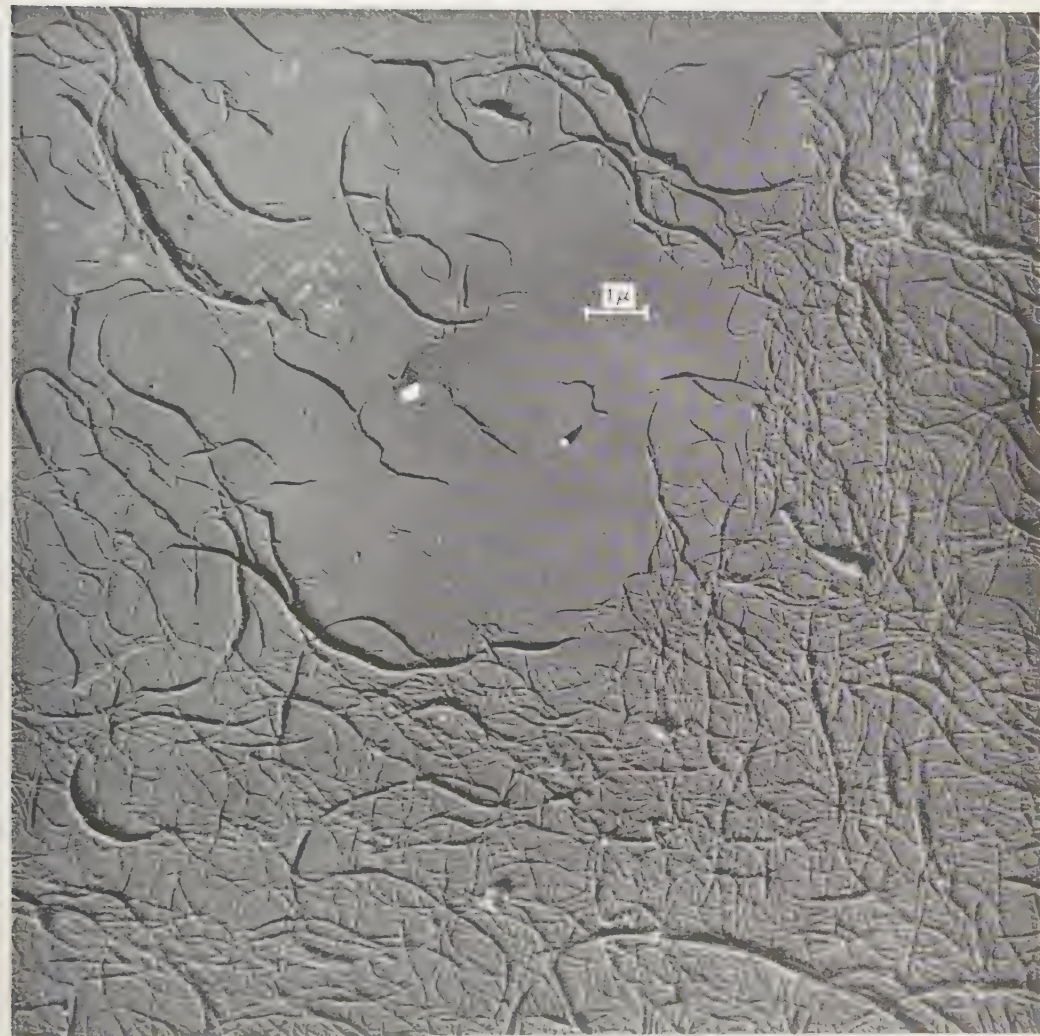
Pseudotsuga taxifolia. Fragment of the secondary cell wall of delignified tracheids, disintegrated in a Waring Blendor and uranium-shadowed.



Pseudotsuga taxifolia. Fragment of the secondary cell wall of delignified tracheids, disintegrated in a Waring Blendor and uranium-shadowed.



Pseudotsuga taxifolia. Portion of the secondary wall before delignification (compare Plate 6), disintegrated in a Waring Blendor and uranium-shadowed.



Pseudotsuga taxifolia. Fragments of the cell wall formed from delignified tracheids during disintegration in a Waring Blender. Uranium-shadowed.

(fibrils 1.4 μ diameter (Ritter 1934), secondary fibrils 0.3-0.5 μ diameter (Seifriz and Hock 1936), dermatosomes (Wiesner 1886), ellipsoid bodies (Farr and Eckerson 1934)). In view of the long thread-like form of the microfibrils which, as suggested above, probably correspond to the "micelles" of early workers, these larger microscopic bodies are to be regarded as consisting of groups of the ultimate microfibrils, which separate under more or less severe mechanical or chemical treatment, and so cannot be regarded as having any significance of their own. Some fragments of the cell wall isolated during the present investigation are shown in Plate 10. The more recent observation by Preston *et al.* (1948) and Frey-Wyssling, Muhlethaler, and Wyckoff (1948) would, however, suggest the existence of submicroscopic structural units of uniform dimensions greater than those of the ultimate crystalline regions deduced from X-ray data (Wardrop 1949). However, the fact that no such fibrils of uniform size were observed during the present investigation indicates that this question requires further study.

In further electron microscopic studies of the cell wall it is proposed to investigate in greater detail the nature of the crystalline regions, and the cell wall organization of cells other than conifer tracheids.

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TREE GROWTH STRESSES

I. GROWTH STRESS EVALUATION

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Summary

Experimental methods for the determination of natural longitudinal stresses in trees are described and the data are used in the development of a mathematical expression for the stress distribution.

Transverse stresses naturally existing within the tree are similarly investigated and the results analysed mathematically.

Another significant class of stresses is shown to result directly from the felling or cross-cutting of a tree.

I. INTRODUCTION

Many difficult problems arise in efficient timber utilization. Not the least of these concern the development of shakes on felling or on subsequent cross-cutting of trees, the tendency of flitches to spring and twist† when sawn from logs and the development of "brittle heart" (Dadswell and Langlands 1934) or similarly degraded wood tissue. Although at least some of these phenomena point to the existence of stresses of considerable magnitude within the tree at the time of felling, little effort appears to have been made to reach an understanding of the forces involved.

Martley (1928) sought to explain observed longitudinal strains (changes in length) in terms of the weight of the tree. Koehler (1933) advanced a number of hypotheses as to the causes of shakes and checks in standing trees. However, Jacobs (1938) was the first to make measurements of strains. Although his experiments were concerned chiefly with strains existing in a direction parallel to the height of the tree, he gave some consideration to changes of dimension in a transverse plane.

Martley (*loc. cit.*) noted a tendency towards a selective alteration in length of a plank cut through the centre of a log. The change occurred immediately after cross-cutting and produced curvature of the face of the plank. Adjustments were such that the maximum length of the plank occurred in the position corresponding to the pith, suggesting that in this area the wood had expanded longitudinally on sawing from the tree. Evidently the release of internal stresses was responsible. In an effort to explain this behaviour, Martley made calculations of the compressions which might result from the weight

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† For definition of terms see Coun. Sci. Industr. Res. Aust., Div. For. Products Trade Circ. No. 15, 1933.

of the tree. He showed that these stresses were unlikely to rise much above a value of the order of magnitude of 300 lb./sq. in. in symmetrical trees, and that a value appreciably lower was more probable. He deduced that such stresses were unlikely to account for the measurable changes in length which had been observed.

Measurements made by Jacobs showed the existence of a longitudinal strain gradient across any radius varying from extension at the periphery to compression towards the pith. The stress equivalent of these strains, when considered in relation to the known strength properties of the timber, suggested to him that some special method of absorption of the applied forces might exist in the zone towards the pith of the tree. He reasoned (1945) that longitudinal tension continuously applied at the periphery could give rise to the observed strain distribution. The strains measured are equivalent to stresses of several thousand lb./sq. in. and are much higher in value than could occur as a result of the weight of the tree. In addition, he pointed out that the existence in pendant eucalypt branches of a stress distribution similar to that of an erect tree is not in accordance with the tree weight hypothesis of the origin of observed strains.

Investigating transverse strains, Jacobs (1939, 1945) showed the existence of peripheral compressions in the circumferential direction. However, in this field his observations were largely qualitative.

Jacob's data on longitudinal strains have been developed and the relationship to transverse strains shown. In addition, a more comprehensive approach to transverse strain evaluation has been attempted.

II. LONGITUDINAL STRESS EVALUATION—EXPERIMENTAL PROCEDURE AND RESULTS

Because the methods of investigation adopted by Jacobs may render interpretation difficult, an experiment was planned to obtain spot confirmation of the magnitudes and directions of strains measured by him; and to study the possible effect of felling and cross-cutting in disturbing the natural distribution of longitudinal strain in a tree.

Green logs of mountain ash (*Eucalyptus regnans* F.v.M.) were chosen. The first of these was 26 ft. long and approximately 23 in. diam. at mid-length. Others were 18 ft. long and from 15 to 24 in. diam. Dial gauges calibrated to read changes in length to the nearest 1/10,000 in. were used for strain measurements.

A parallel-sided slot approximately 2½ in. wide and 18 in. long was cut from the bark into the pith of the log, at about the centre of its length. On the sides of the slot, spikes were driven tangentially to the growth rings, and along lines parallel to the length of the tree, in such manner as to act as reference lengths for the strain gauges. Generally, these reference lengths were approximately 3¾ in. The gauges were supported in position on two main lines—one near the periphery, and one towards the pith.

After initial readings of the gauges had been recorded, the log was cross-cut at positions successively closer to the slot. After each cross-cut, the gauge

readings were noted, and thus the changes in reference lengths determined. An impression of the arrangement of gauges and the method of procedure may be obtained from Plate 1 and Figure 1. Figure 2 sets out some results of strain measurements in the form of two typical strain relief curves.

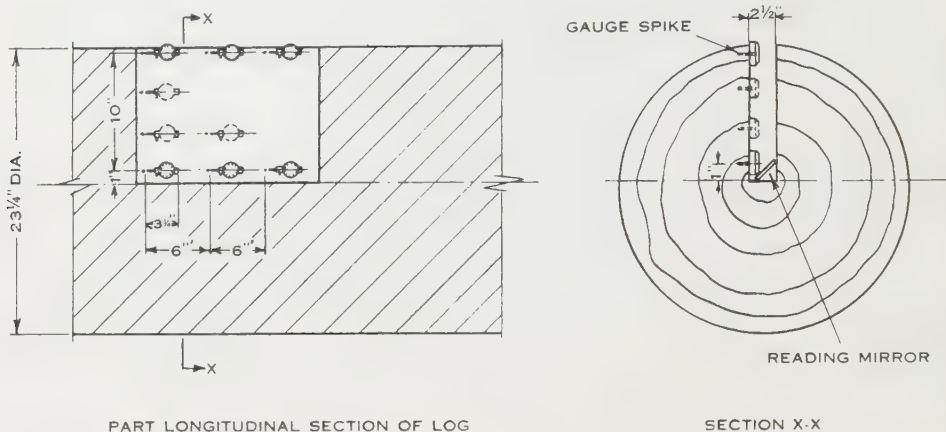


Fig. 1.—Sketch of arrangement of dial gauges for longitudinal stress measurements in trees.

It is not proposed to analyse all aspects of the results at this stage. However, applying the principles of elasticity, it will be appreciated that the removal of longitudinal restraint, which occurs as a result of cross-cutting, allows the relief of the primary longitudinal strains (and their equivalent stresses) in the immediate vicinity of the cut. Obviously, however, there must theoretically be some position remote from the face at which the longitudinal strain is undisturbed. The curves obtained from this experiment indicate that in this instance, significant change of strain did not occur at points distant more than approximately 3 ft. from the cross-cut face.

In addition to the experiment described above, specimens were cut from the log along the wall of the slot and immediately behind each row of gauges. On loading these in a compression testing machine, it was possible to determine the stress equivalent of strains measured by the dial gauges.

Near the periphery, the gauges recorded a shortening over the reference length, as cross-cutting proceeded. This was indicative of a state of tension at this position in an undisturbed tree. The total shortening, up to the stage when the cross-cut was within approximately 1 in. of the end of the first reference length, indicated an equivalent stress somewhat greater than 1,000 lb./sq. in. This is of similar order of magnitude to the figure of 1,200 lb./sq. in. determined by Jacobs on alpine ash (*Eucalyptus gigantea* Hook. f.) and other species.

Near the pith, the gauges indicated a lengthening over the reference distance as cross-cutting proceeded. This was indicative of a state of compression at this position in an undisturbed tree. The equivalent compressive stress was approximately 2,000 lb./sq. in.

The experiment was repeated on two other logs of mountain ash, one approximately 24 in. diam. and the other approximately 15 in. diam. at the gauging section. In each case similar stress distributions were found, though the tensile values were of the order of 2,000 lb./sq. in., and the compressive stress values nearer 3,000 lb./sq. in. In no case was strain detected in a zone distant approximately one-third of the radius of the log from its periphery.

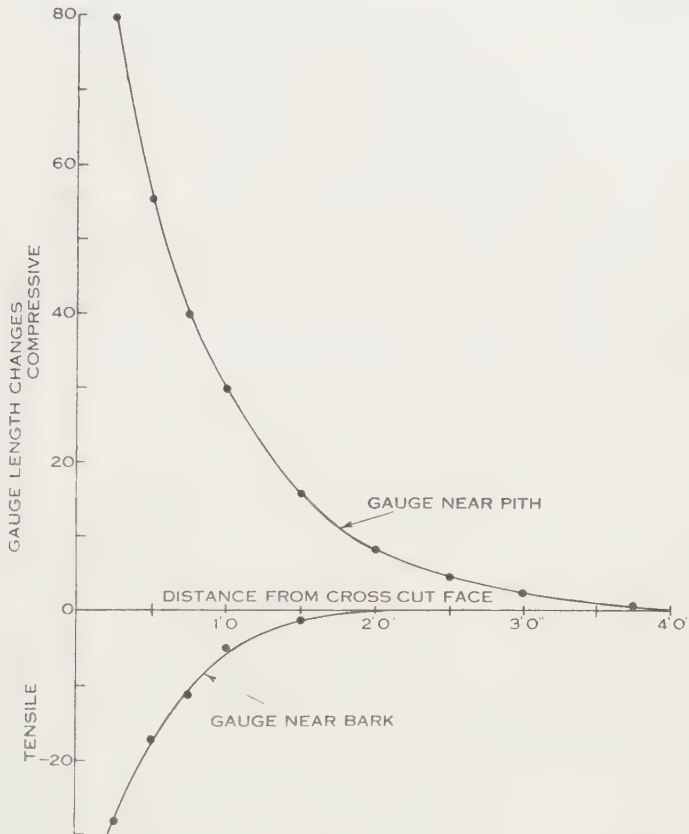


Fig. 2.—Elastic longitudinal strain recovery in trees. Length changes were measured in inches $\times 10^{-4}$; gauge length was approximately $3\frac{1}{4}$ in.; and maximum change of length at failure approximately 100×10^{-4} in.

The compressive stress value of 2,000 to 3,000 lb./sq. in. is considerably below the theoretical amount of approximately 11,000 lb./sq. in. (minimum) on the central 2 in. diam. core, computed for a tree 24 in. diam. and on the hypothesis of longitudinal strain proposed by Jacobs (1945). However, the latter value is in excess of the maximum strength of the wood even on the basis of short duration or standard test loading, and therefore could not be expected. On the other hand, Jacobs's results on residual strains (1939) in

positions towards the centre of the tree reveal similar "low" values. His measurements indicated that the large theoretical strains were not fully recoverable from wood near the centre of trees, and he attributed the cause to long-duration loading. It is interesting to note that in the experiments described here, matched specimens taken from the edge of the slot, and re-subjected to the same strain as had been recovered from them, proved to be strained beyond the elastic limit on the basis of a standard, short-duration test, i.e. they were subject to plastic strain in their natural condition within the tree.

Generally then, these experiments supported the existence of strain patterns identical in type with those indicated by Jacobs's work. Further, the magnitudes of strain measured at various positions within the logs were generally similar to those deduced by Jacobs from the curvature of small strips of wood taken from various positions within the tree.

As pointed out earlier, one of the main purposes of the experiments detailed was to obtain data on the relief of longitudinal strain in the region of a cross-cut face. Jacobs took no account of this phenomenon; however, detailed consideration of the likely effects in the experimental routine followed by him suggest that the errors are not significant in the results used to calculate longitudinal stress development. For this reason, advantage will be taken of his results in subsequent sections of this paper.

III. MATHEMATICAL CONSIDERATION OF LONGITUDINAL STRESSES

Jacobs (1945) reported that the growing sheath of cells in erect stems of alpine ash develops a longitudinal tension which appears to be approximately constant in all diameter classes. Its measured strain of 0.0008 represents an equivalent tension of 1,200 lb./sq. in. Similar strains were measured on trees of other species. He stated that this continuously recurring tension has the effect of imposing a progressive longitudinal compression on the core, the amount imposed by each new sheath of cells (if assumed of unit thickness) being represented by the equation

$$\Delta y = \frac{0.00364}{x^{0.925}},$$

where Δy = the longitudinal compressive strain induced in the entire area of core within the sheath of cells considered, by a unit thickness (radially) of the sheath, and

x = the diameter of the tree or of the growing sheath of cells in inches.

This is equivalent to

$$du = \frac{0.001916}{r^{0.925}} dr,$$

where r = the radius of the growing sheath of cells in inches, and

du = the longitudinal compressive strain induced in the core by a sheath of thickness dr (radially).

In addition, Jacobs (1945) published the curve of progressive longitudinal compression of the core of a tree. This may be obtained by integration of the expression above, whence

$$y_1 = -0.02555 (r_0^{0.075} - r_1^{0.075}),$$

where the negative sign indicates compressive strain,

r_0 represents the outside radius of the tree (inches),

r_1 represents the radius of core used as a datum of strain calculations, that is, the radius at the point for which a valuation of the strain is required (inches),

y_1 represents the compressive strain imposed on the core at radius r_1 by the sheaths external to it.

This equation may be used to determine the compressive strain imposed at any radius r_1 by successive sheaths of cells external to it, as the tree grows from radius r_1 to radius r_0 . However, as each sheath of cells initially develops a tensile strain t_0 , the first increments of compressive strain are counterbalanced, and the total residual compressive strain on any sheath is that determined from the equation above, less the equivalent of t_0 . It can be shown, therefore, that the longitudinal strain theoretically existing in a sheath of cells at any radius r_1 is given by the equation

$$y_1 = -0.02555 (r_0^{0.075} - r_1^{0.075}) + t_0. \quad . \quad . \quad (1)$$

Because of the phenomenon of plastic relaxation of stress, the full theoretical strain, though possibly applied to a particular group of cells considered, will never be elastically recoverable from it. Such relaxation must tend to be greater in the more highly stressed parts of the tree than elsewhere. This is partly on account of the higher stress and partly because of the longer duration of load on the older wood in this area. The effect of the relaxation is to absorb some of the strain energy in a form which does not allow immediate elastic recovery. As a consequence, immediately recoverable longitudinal strain will be less than that shown in equation (1). Nevertheless, the equation serves as a useful basis of strain analysis.

Though the compressive strain imposed on the core by each sheath of cells varies inversely with the radius of the sheath, the total effect appears to be uniformly distributed over the core. Thus successive sheaths of growing cells cause a progressively higher value of compressive strain to be developed in the region towards the pith. Consequently, as the diameter of the tree increases, the strain ultimately reaches such a value that the limit of purely elastic strain is passed (Jacobs 1945). From this point plastic flow commences and some permanent and irreversible adjustment occurs in the fundamental structure of the wood. In this regard it should be realized that some plastic flow may occur in wood at relatively low stresses (much less than the usual value of elastic limit based on short duration tests in a testing machine). The seriousness of this "flow" would depend upon the duration of loading as well as the intensity of stress.

Although alpine ash was the only species investigated in detail by Jacobs, specimens from a considerable number of other pored species were measured. Without exception these showed strain gradients similar in nature to that of alpine ash.

IV. STRESSES IN A TRANSVERSE DIRECTION

Frequently, particularly in pored species, checks or shakes develop from the pith outwards, immediately after cross-cutting both green and relatively dry logs. These shakes cannot be explained as drying checks, but are obviously manifestations of stresses within the living tree. Despite their significance in timber utilization, no satisfactory explanation of the phenomenon is available.

Koehler (1933) considered some of the explanations which have been suggested. He attributed the shakes and cracks to stresses operating at right angles to the grain, that is, in a transverse plane. He observed that, for many species, if a disc is cut from a green tree trunk the outside portion is in circumferential compression. He reasoned that such peripheral compression is associated with radial tension, and that this combination could be responsible for the opening up of heart shakes.

Jacobs (1939) made qualitative and quantitative demonstrations of the existence of a continuous strain gradient across the transverse section of a disc cut from a green log. From one set of experiments he concluded that a general state of circumferential compression in a direction tangential to the growth rings existed throughout the disc. Later experiments (1945) suggested to him that the central zones of the discs were in a state of tension.

Because of this anomaly, which appears to have been caused by interactions of radial and tangentially directed strains, new experiments have been carried out. The primary purpose of these was to obtain information on stresses causing the development of shakes in the ends of logs. The object was to demonstrate the nature and measure the value of both circumferential or ring stresses, and those directed along the radius of a transverse section of a tree.

With this in mind, it was considered desirable to establish primarily that any circumferential and radial stresses investigated by means of sections cut from a log were truly representative of similar stresses present in the standing tree. In addition, further experiments have been carried out to show the direct effect of longitudinal strains at any point within a log in producing corresponding transverse strains and also the effect of release of longitudinal strain energy at a cross-cut face in producing transverse strains across that face.

(a) *Experimental Procedure and Results*

(i) *Reliability of Laboratory Measurements.*—For the purpose of establishing whether strains determined from laboratory measurements on discs cut from logs were truly representative of conditions in the growing tree, an experiment was conducted on trees of mountain ash and messmate stringybark (*Eucalyptus obliqua* L'Herit.). Part of this experiment was carried out on

living trees in the forest. The trees were subsequently felled and investigated both in the forest and in the laboratory. In the forest, the experimental procedure was to select two trees of each species having diameters within the capacity of gauges available ($1\frac{1}{2}$ in.). A short length of bark was removed carefully from the full periphery, at several positions. Heights of the gauge positions were approximately 18 in. and 3 ft. above the proposed level of the felling cut. Right-sections were drawn around the tree at the positions chosen, and hemispherical-headed nails were driven in at the ends of two diameters at right angles.

Measuring apparatus consisted of a dial gauge set in one end of a stiff, adjustable frame. A hollowed-out seat for the large, hemispherical-headed nails was provided at the end of the frame opposite to the gauge. With the fixed seat held in position over one nail and the gauge contacting the head of the diametrically opposite nail, as in Plate 2, Figure 1, it was possible to make comparative measurements of each diameter to the nearest $1/1000$ in.

After measuring diameters with the tree standing, the tree was felled and again measured. No change in diameter of the gauging positions was observed. Logs, each 7 ft. long and including the gauging positions, were cut from the trees. After re-measurement had established no diameter change, they were transported to the laboratory and measured a day after felling. Still no change was detectable. Finally, discs containing the reference points were removed from the logs, using a technique to be described in detail later.

In these experiments no significant change of diameter occurred as a result of felling or any of the subsequent operations, except in one instance, in which a felling check passed through the gauge section. As any change in transverse stresses between the location in the growing tree and in the log at the laboratory would tend to show as variations in diameter measurements, it was concluded that no significant change occurred, and that laboratory measurements on these logs provided a fair method of assessing transverse stresses in the undisturbed tree.

(ii) *Radial Strain Experiment.*—Discs were cut from a green log of *E. regnans*. These were approximately 27 in. in diameter and 2 in. thick, with parallel, right-section faces. Faces were dressed smooth. A "V" was drawn on the face, with the apex at the pith, and the open end approximately 4 in. wide. A similar "V" was drawn to the side of the first. Specially prepared $\frac{3}{4}$ in. long copper nails were set in radial lines approximately $\frac{1}{2}$ in. inside the "V" lines, and 2 in. apart in the radial direction. These nails were driven firmly into pre-drilled holes. Their heads carried finely etched, crossed lines. The arrangement is shown in Plate 3. A measuring microscope with graduations of approximately $1/25,000$ in. was used for distance measurements.

After preparation of the disc, measurements were taken between each pair of nails. The wedge-shaped sections were then cut from the disc as in Plate 3 (*b*). It was considered that the distances between nails, before and after freeing the wedges from the restraint of the disc, represented the strained and the unstrained lengths respectively of the wood between. To check that

radial strain was as completely released as possible, one wedge was further divided as shown in Plate 3 (c). Care was exercised during the whole experiment in an endeavour to keep the moisture content of the wood constant.

Adjacent side-matched specimens of wood, taken from the disc and parallel to the direction of radial strain, were tested separately in tension and compression. From these tests, the stress equivalents of measured radial strains were determined.

The distance between nails on radial lines became less as the restraint was removed. This indicated a state of radial tension in the original disc. There was no significant effect of the subdivision of the wedge. Actual differences in measured spacings before and after restraint removal are shown in Table 1. The gauge length was 2 ± 0.02 in. The stress equivalent of the average strain measured was approximately 150 lb./sq. in. radial tension.

TABLE 1
RADIAL STRAIN IN TREE
(*E. regnans*, 27 in. diam.)

Marker Pairs	Reduction in Marker Spacing, Before and After Stress Relief (in. $\times$ 1/1000)					Average Radial Tensile Strain
	Row A	Row B	Row C	Row D	Average	
1-2	2.2	3.9	2.0	2.7	2.7	0.0013
2-3	3.0	3.3	1.7	2.2	2.7	0.0013
3-4	2.6	2.5	2.5	2.0	2.4	0.0012
4-5	3.1	2.4	3.1	2.6	2.8	0.0014
5-6	2.2	2.8	3.0	1.7	2.4	0.0012
6-7	1.0	1.3	1.5	0.8	1.2	0.0006

(iii) *Circumferential Strain Experiment*.—In this experiment, trees ranging in diameter from 5 in. to 13 in. were investigated. Laboratory tests were made on logs 7 ft. long. These were kept green and at approximately constant temperature and moisture content during test.

Species investigated included *Eucalyptus regnans* F.v.M.—3 logs; *Eucalyptus obliqua* L'Herit.—2 logs; *Eucalyptus sieberiana* F.v.M.—1 log; *Eucalyptus australiana* Baker and Smith—1 log; *Acacia dealbata* Link—1 log; *Acacia melanoxylon* R.Br.—1 log; and *Pinus radiata* D. Don—2 logs. Measuring frames were of the type shown in Plate 2, Figure 1, and calibrated to measure differences of 1/1000 in. Three specimen discs were cut from each log—one at the centre of the length, and the others at approximately a quarter of the length from the ends. A special technique was required to ensure that major heart shakes did not develop within the discs on cross-cutting, as these would render subsequent measurements valueless. A comparison of discs cut with, and without, the adopted technique is shown in Plate 2, Figure 2— M_4 and M_5 showing the minor shakes associated with the technique, and M_3 showing the normal shakes for the specimen (other specimens developed even larger shakes). A description of the technique and its significance will be given later in this paper.

The procedure of the experiment consisted in setting hemispherical-headed nails around a right-section of the log, and at opposite ends of two diameters at right angles. A disc containing these nails, and approximately $1\frac{1}{4}$ in. thick, was cut, and the diameters across pairs of nails measured accurately with the special comparator gauges. A hole was drilled about $1\frac{1}{2}$ in. from the periphery, and a saw cut made approximately along the line of the growth rings so that the outer annulus could be removed from the core as in Plate 2, Figure 2. In every instance the annulus expanded, showing that the wood composing it had been under a state of circumferential compression in the disc. The changes in diameter were measured, and results are set out in Table 2.

A load-strain curve was determined from an experiment on matched specimens of mountain ash (*E. regnans*). This indicated that the mean measured strain in the annulus was equivalent to a ring compressive stress of approximately 300 lb./sq. in.

It is of interest to note that Jacobs (1945) quoted a comparable figure of 360 lb./sq. in. for *E. gigantea*. When allowance is made for the variability of the material, the species, and also the width of the annulus, the agreement is close.

(iv) *Direct Effect of Longitudinal Forces in a Transverse Plane.*—Measurements on trees of *E. regnans* and *E. obliqua* indicate that transverse stresses are not significantly altered in conversion of trees to logs. Further, it may be deduced that longitudinal stress arising from the weight of the tree has no significant effect in a transverse plane.

Though no longitudinal stresses can exist at the cross-cut face, experiments have shown their existence within the log. The problem thus becomes one of relieving the longitudinal stresses at the gauge section in such a manner that no confusing interactions are induced, and of measuring any effect. To help achieve this, the special technique previously mentioned was used to prevent the usual formation of heart shakes.

In this experiment the following species were examined: *E. regnans*, *E. obliqua*, *E. sieberiana*, *E. australiana*, *A. dealbata*, *A. melanoxylon*, and *P. radiata*. Measurements of diameter in the log and on the discs after cutting showed no diameter change, i.e. within the accuracy of measurement ($1/1000$ in.) no change of strain was measurable, even on the largest diameter log. Thus the effect on the diameter of any variation of strain which may have occurred as a result of the relief of longitudinal stress was less than $1/(1000 \times \text{diam.})$, or less than a diametral (or circumferential) strain of $1/10,000$. The equivalent stress would be less than 30 lb./sq. in., approximately, in the transverse plane.

(v) *Effect of the Release of Longitudinal Strain Energy in Producing Transverse Strains.*—The general plan of the experiment has been described in connection with the measurement of longitudinal strains and the arrangement is shown in Figure 1 and in Plate 1. Between the banks of gauges at the periphery (tension zone) and near the pith (compression zone), several other gauges were placed.

TABLE 2
DIAMETRAL STRAINS IN TREES

Species	Log Number	Disc No. 1				Disc No. 2				Disc No. 3				Mean Diametral Strain $\times 10^4$
		Diameter A		Diameter B		Diameter A		Diameter B		Diameter A		Diameter B		
		Approx. Diam. (in.)	Diam. Change ($\frac{\text{in.}}{1000}$)	Strain $\times 10^4$	Approx. Diam. (in.)	Diam. Change ($\frac{\text{in.}}{1000}$)	Strain $\times 10^4$	Approx. Diam. (in.)	Diam. Change ($\frac{\text{in.}}{1000}$)	Strain $\times 10^4$	Approx. Diam. (in.)	Diam. Change ($\frac{\text{in.}}{1000}$)	Strain $\times 10^4$	
<i>E. regnans</i>	1 ^o													
	2	11½	14	12	12	12	7	13	7	5	11½	13	11	12
	3	5½	6	10	6	6	2	6½	6	9	5½	2	4	8
<i>E. obliqua</i>	1	10½	13	12	11½	13	12	10½	12	11	10½	11	10	11
	2	9	8	9	10	22	22	9½	10	11	9½	10	10	13
<i>E. sieberiana</i>	1	8½	8	9	9½	22	23	8½	9	11	10	19	19	21
<i>E. australiana</i>	1	7½	8	10	7½	8	11	7½	11	15	8	13	16	10
<i>A. dealbata</i>	1	5½	7	12	6	3	5	6½	5	8	6	5½	12	21
<i>A. melanoxylon</i>	1	5½	3	5	6	5	8	6	7	12	6	5½	3	6
<i>P. radiata</i>	1	7½	5	6	7½	7	9	8	6	8	7½	6	8	5
	2	7½	5	7	7½	4	5	7½	8	11	7½	7	10	8

^o *E. regnans* log No. 1 was cut before a satisfactory technique had been developed. Severe heart shakes caused fracture of the annulus during removal.

For the two main banks of gauges, curves of gauge length changes, in relation to distance from the cross-cuts, are shown in Figure 2. The position of gauges between the two main banks was so chosen as to coincide with the theoretical zero strain position. The theoretical basis of strain calculation was confirmed in each case, in that gauges so located did not show any significant strain changes during the whole process of strain (and stress) relief.

An analysis of the results of this experiment suggests that strain adjustment consequent to stress relief may be mathematically expressed by a function of the nature

$$\log Y = A + Bx + Cx^2,$$

where Y represents the movement parallel to the length of the log, of a point at a particular radial position and originally on a right cross section plane,

x represents the distance of the right cross section considered from the cross-cut face,

A , B , and C are constants.

In reference to the relief of tensile stresses, the constants are capable of determination with much more exactitude than applies to the relief of compressive stresses. This is because the gauge limit spikes in the compression area tend to cross growth rings, and are therefore subject to strain reactions which are not uniform along their lengths. A typical tensile strain relief curve is

$$\log (-Y) = 2.80 - 0.11x + 0.0020x^2,$$

where x is in inches and $-Y$ represents a reduction in length measured in inches $\times 10^{-5}$.

The stress equivalent of strains noted may be determined from stress-strain curves for specimens of the material placed in a testing machine. From these, the equivalent residual stress values for the original tree or log may be calculated. The use of strain values and the species mean modulus of elasticity of the timber, determined by standard testing procedure, as an alternative method of stress assessment is unsatisfactory, particularly in the high-intensity compression zone.

The curves show that, at all positions between the cross-cut and the gauging point, length changes of varying degree occur in the wood structure. These changes indicate corresponding variation of stress over the volume of wood considered. In turn, this implies changes in the applied forces and the "work done" or "energy" absorbed within the material. The effect of cross-cutting is to reduce restraint at that section on the internal forces in the log; that is, it allows some of the strain energy originally held within the wood to be released. The experiment also shows that strain energy is released by the various wood elements throughout the length of the log affected, in a regulated and definite manner. The degree of release at various points indicates that this energy is transferred to the cross-cut face.

To date, results from only three logs of mountain ash are available. Although it might be anticipated that similar strain reactions will occur through-

out this and other species, the quantitative expression of the results may vary with the species, the size of tree, age, etc. It is proposed to investigate this phenomenon further, but meanwhile, from these limited results some impression of the total volume of strain energy released at the cross-cut face may be obtained, and an approximate calculation of its value made.

That the release of longitudinal strain energy at the cross-cut face is of practical importance may be readily shown by a separate experiment. The cross-cutting technique mentioned earlier will be used to illustrate the point.

During the previous experiment, it was noted that, immediately after each new cross-cut was made, large heart shakes opened up in the newly exposed faces of the log. If the section cut off was a few feet or less in length, the shakes in the new face of that piece were less severe than those in its opposite end, and in the new face of the parent log. Taken in conjunction with the strain energy deductions discussed above, it led to the hypothesis that the development of shakes resulted directly from strain energy movements, and that control of strain energy release may give control of the development of heart shakes.

In this experiment and in many other observations, it was noted that, immediately after cross-cutting, shakes split the log for a distance generally not more than a few inches in the direction of the length except in very severe instances. It was thought that at the plane at which splitting stopped, probably the accumulation of strain energy was less than the critical splitting value for the log. On the basis of this hypothesis, control or measuring sections, a few inches thick in the direction of the length, were marked on a number of logs. The diameters varied from 4 to 13 in., and the positions selected were between 1 and 2 ft. from an existing face (depending upon the tree diameter). Short sections were cut away successively from both ends of the log, retaining the control section approximately central. As discs were removed from the ends, the strain energy from the remainder tended to "flow" to the new ends, causing shakes to develop. However, as the control was approached the shakes developed were smaller, and by the time the control section was isolated, virtually all longitudinal strain energy had been previously released from it and absorbed in cracking reject parts of the log. Consequently, the control sections were almost entirely free of shakes. The detailed result of the application of the special technique to three sections of one log, approximately 8 ft. long and 13 in. diam., is shown in Plate 4. The discs at the end of each line show the severe cracking or development of heart shakes resulting from normal cross-cutting, or non-control of longitudinal strain energy dispersion. The reduced development of shakes in discs towards the central control disc in each line is obvious.

From the description of procedure it will be obvious that only longitudinal strain energy dispersion is different in the different discs, and that this alone must account for the variation in development of shakes. The importance of this effect is therefore considerable. Further, reference to the other experiments described will show that cross-cutting alone does not disturb transverse

strains. The technique proposed makes this more positive by ensuring that transverse strains are not relieved as a result of cracking of the disc, and it is the only satisfactory method of preparation of sections for transverse strain measurements.

(b) *Discussion of Transverse Strain Experiments*

Under the conditions in which transverse strains have previously been investigated, nominally all the longitudinal stresses have been relieved at the cross-cut face. Thus the reactions in a transverse plane during the relief of longitudinal stress may be proposed as responsible for bringing transverse strains into existence.

If it is supposed that readjustment of cell cross sections on relief of longitudinal stresses is a valid explanation of the development of the measured transverse strains after cross-cutting, then it follows that such transverse strains did not exist in the standing tree. Occasionally, the appearance of heart shakes after large trees have been felled suggests that shakes were present in the standing tree. As the shakes are indicative of a high level of transverse stress, it appears that such stress develops in growing trees, that is, before longitudinal stresses are substantially relieved. In such trees it is difficult to visualize the occurrence of any sudden or substantial relief of longitudinal stress which might account for the development of this transverse stress and consequently the shakes. Therefore, another primary source of transverse strains is indicated.

It may be of value, however, to consider the likely effects of relief of longitudinal stress. Longitudinal compressive stresses can be built up only after the wood structure has reached a mature and elastic condition. Hence, any longitudinal stresses which are ultimately relieved by cross-cutting are equivalent to those which have been built up during growth, less any which have been relieved by plastic adjustment in the cell walls. Thus the general effect of a reduction of longitudinal stress to zero would appear to be a corresponding removal of transverse strains.

Here, however, the problem has been over-simplified, in that the longitudinal stresses are developed as the tree grows, and the consequent reactions tend to produce ever higher intensities towards the pith. Therefore, towards the periphery of the tree, new cells are subject to relatively small longitudinal stresses and relatively small induced cross-sectional strain when the central part of the tree is comparatively highly strained in the transverse direction, as a result of the greater longitudinal stress. Thus, considering the transverse plane only and assuming transverse restraint still exists, the conditions under which the peripheral cells are free from longitudinal strain would not coincide with those for an unstrained state in cells towards the pith. It is clear then that apparent total relief of longitudinal strain in a disc representing the full cross section of the tree will not remove all transverse strain resulting from such primary longitudinal strain.

Tests made on specimen trees of *E. regnans*, *E. obliqua*, *E. sieberiana*, *E. australiana*, *A. melanoxylon*, *A. dealbata*, and *P. radiata* appear to indicate that,

for these species, no substantial change in the over-all cross section of the tree occurs as a result of felling, or cross-cutting right up to the face of the section measured. As controlled cross-cutting does not affect transverse strains, it may be deduced that the relief of longitudinal strains by this means does not produce any change in tree diameter—a result which might have been anticipated on consideration of the Poisson ratio effect. For stability, the total longitudinal tensile and compressive forces acting over any cross section of a tree must be balanced, and therefore the gain in area of cross section of cells as a result of relief of tensile forces would balance the reduction in area of cross section of cells as a result of relief of compressive forces. Despite this lack of significant change in area of a disc on relief of longitudinal strain, and under the actual conditions of transverse restraint, the result may still be compatible with the supposed development of transverse strains; but such strains must be appreciably less in value than those present under actual growth conditions. Therefore, it must be considered whether these residuals are sufficient to account for the noted expansion of an outer annulus separated from the core of a disc.

For example, if a tree of mountain ash is considered, and the total original longitudinal stress is of an average value of 2000 lb./sq. in. over the annulus (this is probably more severe than any practical case), and it is assumed that the elastic moduli for the material are 2,000,000 and 70,000 lb./sq. in. in the longitudinal and circumferential directions respectively, and that the corresponding Poisson's ratio is equal to 0.45, then:

$$\text{maximum longitudinal strain} = \frac{2,000}{2,000,000} = \frac{1}{1,000},$$

$$\text{resulting transverse strain} = 0.45 \times \frac{1}{1,000},$$

$$\text{equivalent stress} = \frac{0.45}{1,000} \times 70,000 = 31.5 \text{ lb./sq. in.}$$

Thus the maximum transverse stress which might arise from longitudinal stress in the wood considered would be little more than 30 lb./sq. in. It will be noted that this stress equivalent is approximately one-tenth only of measured peripheral stresses in discs, and as the actual stress from the longitudinal stress residual, rather than the original total stress, is probably much smaller still, its effect is not likely to be significant. Thus there must be some other primary cause of the development of transverse stress.

In addition to the adjustment in cell cross section discussed above, there is another transverse straining effect of the relief of longitudinal stresses. The process of strain development with growth produces a condition of differential longitudinal strain across any diameter. Then if the tree is cross-cut, all longitudinal strain is relieved at the cross-cut face. Also, partial relief occurs at positions beyond the face. This relief requires each cell to adjust its length (as well as cross section) in accordance with the strain previously existing in it. Thus the most highly strained central fibres elongate a greater amount than cells towards the periphery. Cells near the periphery, which are in ten-

sion, reduce their length. The effect of the length reaction is to convert an originally flat cross-cut to a dome shape. This is confirmed by the measurement of the longitudinal length adjustments resulting from cross-cutting. It has been demonstrated, too, that this process of transverse strain generation may produce strains of a value sufficient to cause heart shakes.

V. MATHEMATICAL EVALUATION OF TRANSVERSE STRAINS

For simplicity, the total transverse stress distribution on any cross section can be taken as made up of:

(a) Transverse stress distribution arising from primary circumferential stresses in the growing sheath of cells.

(b) Secondary transverse stresses which develop from changes in cell cross sections induced by primary stresses existing in a longitudinal direction.

(c) Secondary transverse stresses resulting from the change in length of cells at, and near, a cross-cut face.

It should be clear that the first class of stresses are assumed present in the growing tree, and are primarily independent of cross-cutting. The second class also will be present in a growing tree, but would tend to be relieved by cross-cutting. The third group of stresses can arise only when there is opportunity for substantial longitudinal stress reduction. Though some degree of cross-sectional adjustment may occur in a standing tree as a result of partial inelastic reaction to longitudinal stress, such as plastic action or possibly structural failure of individual fibres, perhaps the most important practical case arises with relief of the residual longitudinal stress on cross-cutting. However, because of the balanced nature of the longitudinal stresses across every cross section, and because of the resistance to differential movement between fibres, no substantial effect of fibre length reaction can occur except near the cross-cut face.

(a) Transverse Stresses Arising from Primary Circumferential Stresses

As a result of qualitative experiments on a considerable number of trees and a variety of species, Jacobs (1945) has shown the existence of circumferential strain of appreciable magnitude. On the basis of these experiments, he has suggested the hypothesis that this strain arises in the growing sheath of cells and is continuously renewed at the same value in each new sheath of cells—a phenomenon similar in principle to that which appears to apply to longitudinal strains. This hypothesis is supported by the results shown in Table 2. It is proposed to develop an expression which will represent the transverse stress at any point in a cross section as a result of such primary circumferential strain and its related stress.

Jacobs's experimental work on longitudinal strains leads to the conclusion that each increment of strain at the periphery impresses an approximately uniform strain reaction upon the core. If this is so for primary longitudinal

strains, it appears very likely that a similar law would regulate the effect of primary transverse strains. This assumption will be made as a basis for calculation of a theoretical strain distribution.

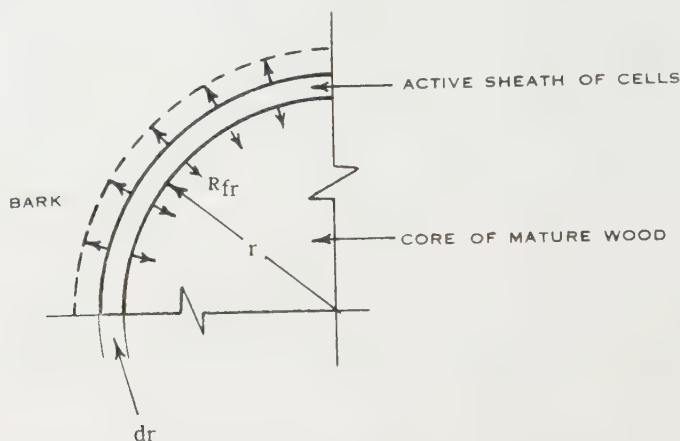


Fig. 3.—Portion of the cross section of a tree trunk.

In Figure 3, it is supposed that the sheath of cells of thickness dr and radius r is the "stress active" portion of the tree, producing circumferential and radial stresses. Through some agency not yet established, this sheath shows a tendency to expand circumferentially, and consequently in diameter. Such expansion is resisted by the attached central core, or enveloped portion of the tree, and to some degree by the bark. The effect of this restraint on free movement must be to produce compression in the active sheath, and at the same time radial tension in the core.

If a radial stress R_{fr} is imposed on the core at radius r , and causes equilibrium of the whole transverse section, then this stress is balanced at the annulus-core interface. Also, if the annulus, or sheath of cells, is regarded as a thin cylinder, then

$$\begin{aligned} \text{circumferential stress in the active sheath} &= \frac{\text{radial stress} \times 2 \times \text{radius}}{2 \times \text{thickness of annulus}} \\ &= \frac{-R_{fr} \times r}{dr} \quad \dots \quad (2) \end{aligned}$$

The negative sign indicates a compressive stress.

On the assumption that primary strains arising at the periphery impose a uniform strain on the core, then the radial strains must be equal at all points along a diameter. This hypothesis is supported by the measurements of radial strain shown in Table 1. With the exception of one measurement taken near the pith, which was possibly affected by a small check, strains were substantially equal at all points.

Now if C represents the strain (or change in length per unit length) in the circumference at any radius r ,

$$\begin{aligned}\text{the unstrained length of the circumference} &= 2 \pi r, \\ \text{the change in length of the circumference} &= 2 \pi r C, \\ \text{hence the strain on the radius} &= C.\end{aligned}$$

Thus at any radius, the radial strain is equal to the circumferential strain. Consequently, on the hypothesis assumed above, circumferential strains, as well as radial strains, must be equal at all points along the core diameter.

Now from the theory of elasticity, the relationship between stress and strain is such that

$$\text{radial strain} =$$

$$\frac{\text{radial stress}}{\text{Young's modulus for strain in a radial direction } (= E_R)},$$

and circumferential strain =

$$\frac{\text{circumferential stress}}{\text{Young's modulus for strain in a tangential direction } (= E_T)}.$$

Hence, as circumferential and radial strains are equal,

$$\begin{aligned}\text{imposed circumferential stress} &= \text{radial stress} \times \frac{E_T}{E_R} \\ &= R_{fr} \times \frac{E_T}{E_R}, \quad . \quad . \quad . \quad (3)\end{aligned}$$

and is constant along the diameter.

Comparing equations (2) and (3), if the circumferential compressive stress in the active sheath is represented by the quantity

$$- \frac{R_{fr} \times r}{dr},$$

then a tensile stress of value

$$+ R_{fr} \times \frac{E_T}{E_R}$$

is impressed at all points along the diameter of the core, and in a tangential direction. Similar increments of stress imposed by each successive sheath of cells on the peripheral side are added to the stress primarily existing in the particular sheath, when it represented the periphery of the tree. Thus it is seen that if each new sheath of cells generates an initial compressive stress, each succeeding sheath imposes a tensile stress on the first. Consequently, the tendency is to reduce the intensity of the original compression, and ultimately to convert it to a tensile stress.

If the circumferential stress in the peripheral sheath of cells has the value $-S$ and it is assumed, on the basis of the experiments described, that a stress of the same value is generated in each succeeding sheath, independently of the diameter of the tree at the time, then from equation (2),

$$-S = - \frac{R_{fr} \times r}{dr},$$

$$\text{or } R_{fr} = \frac{Sdr}{r} \quad . \quad . \quad . \quad . \quad . \quad . \quad (2a)$$

Substituting this value of R_{fr} in equation (3), it may be seen that the radial stress on each stress active sheath of radial thickness dr imposes on the enclosed wood a uniform circumferential tensile stress

$$= \frac{E_T}{E_R} \times \frac{Sdr}{r}.$$

The effect, as the diameter increases from radius b to radius a , is to impose across the core of radius b a circumferential tensile stress

$$\begin{aligned} &= \int \frac{aE_T}{bE_R} \cdot \frac{S}{r} dr, \\ &= S \frac{E_T}{E_R} (\log_e a - \log_e b), \\ &= 2.3S \frac{E_T}{E_R} (\log_{10} a - \log_{10} b). \quad (4) \end{aligned}$$

When the sheath of cells at radius b was originally laid down, it was subjected to an initial compressive stress of value S . Hence at radius b the net tensile stress finally developed in a sheath of cells

$$= 2.3S \frac{E_T}{E_R} (\log_{10} a - \log_{10} b) - S. \quad (5)$$

This expression gives the ring stress at any radius b in the cross section of a tree of diameter $2a$. The stress is directly attributable to an initial, and continuously recurring, circumferential compressive stress in the peripheral sheath of cells.

(b) Secondary Transverse Stresses which Result from Changes of Cell Cross Sections Induced by Primary Longitudinal Strains

A primary longitudinal strain in a tree will produce strains in two directions at right angles—circumferential and radial. Because of the approximately circular form of the tree cross section, and the nature of the longitudinal strain distribution, these strain effects interact.

For simplicity, the general effect of circumferential and radial strains induced by the longitudinal strain in any particular sheath of cells within the tree cross section will be considered separately. Later, the effects of all such sheaths will be combined to obtain the full strain pattern in a transverse section across the tree. A circumferential strain produces a change in the circumference of the sheath equal to the sum of the changes in width in this direction of all the individual cells. A corresponding change in diameter must occur.

Any such sheath of cells, however, is not an independent entity, being attached to other similar sheaths of cells towards both pith and periphery. Thus the rigidity of all other cells restrains the free adjustment of position of the sheath to accommodate the direct tangential effects of longitudinal strain. Consequently, the circumferential strain in the sheath is less than that of an independent sheath.

The difference between "potential" and actual strain in the sheath is balanced by the imposition of radial forces on the core and the outer annulus. In turn, the radial forces upon the core will produce ring stresses as well as radial stresses therein. Also, the forces applied to the annulus will have an effect in the two directions.

Similarly, strains which have a direct component in the radial direction will not have freedom of adjustment. Consequently the residual radial component strain will be less than in a free sheath of cells, and additional strains will be imposed on the outer annulus and the core.

So far discussion has been confined to the manner of production of transverse strains arising from the longitudinal strain in one sheath of cells only. In any case of practical significance, there are many thousands of such sheaths, all producing their complex distribution of strain, and therefore all interacting with one another. Further, this complex system exists quite independently of any system of primary transverse strains, such as that envisaged as arising in the peripheral sheath of cells. Because of the complexity of this problem for a longitudinal strain pattern which is variable across the transverse section, a detailed mathematical solution has not been attempted herein. It is possible, nevertheless, to obtain an impression of the limits of the effect of longitudinal strain in a transverse direction.

Jacobs (1945) has shown that the maximum strain which is recoverable from the core of a tree is equivalent to a stress of approximately 2000 to 3000 lb./sq. in. This is supported by experiments described herein. Adopting the values of elastic constants already used, an approximate calculation may be made as follows:

$$\begin{aligned}\text{longitudinal strain} &= \frac{3,000}{2,000,000} = \frac{1.5}{1000}, \\ \text{resulting transverse strain} &= 0.45 \times \frac{1.5}{1000} = \frac{0.67}{1000}, \\ \text{equivalent ring stress} &= \frac{0.67}{1000} \times 70,000 \\ &= 47 \text{ lb./sq. in.}\end{aligned}$$

Therefore, if the full cross section were uniformly compressed with the maximum elastic, longitudinal strain existing in the tree, the maximum ring tension developed would be approximately 50 lb./sq. in. Actually, the highest intensity longitudinal strains exist near the centre of the tree, and the effect of the lower-strained outer area must be to reduce the corresponding maximum ring stress on the core, possibly to a value of the order of 30 lb./sq. in. (tension).

Such an equivalent stress might be possible in a growing tree, or remote from a cross-cut face. At a cross-cut face, the longitudinal stress is fully relieved. However, because the peripheral cells are differentiated and matured at a time when the core is already highly strained, subsequent removal of all longitudinal strain effect on cell cross sections over the full transverse section

of the tree is not possible. A likely effect in the transverse plane would be a reduction of the tensile strain in the core, with a possible slight increase of the peripheral circumferential compression. If then, the final transverse effect is a maximum residual ring stress of the order of 20 lb./sq. in. in a cross-cut disc, it is small compared with peripheral, circumferential compressions of the order of 300 lb./sq. in. Such compressions have been measured in experiments described herein and by Jacobs (1945). Further, it has been shown theoretically that peripheral compressions of the magnitude of those measured would tend to induce ring tensions much greater than appear to result from primary longitudinal strains.

(c) *Secondary Transverse Strains Resulting from a Change in Length of Cells at, and near, a Cross-cut Face.*

It has been illustrated experimentally that, if a log is cross-cut, the primary longitudinal strain is fully released at the face, and partially released for some significant distance beyond it. It is likely that the length over which significant release of strain occurs depends on the species, the diameter of the tree, the value of the "undisturbed" strain at the cross-cut section considered, and the mechanical properties of the wood in the particular specimen.

Simultaneously with the longitudinal strain variation, there is a corresponding length adjustment in the fibres or wood cells. Movement occurs in the direction of the cross-cut face, and the total deflection of any point on the face is the sum of the separate and varying length adjustments over successive cells beyond the face. This deflection is proportional to the area under a curve plotted to show the relation between longitudinal strain relief and distance from the cross-cut face (Fig. 2). Thus, the area under the curve represents a certain volume of "strain energy" released at the cross-cut face, and at a certain radial distance from the pith.

Because of the variation of longitudinal strain over the cross section of a tree, the adjustments of cell length distort an originally flat face into a "dome" shape. As the length of a curved surface is greater than that of a straight line joining its ends, some strain is induced when such a surface is developed from an originally flat one subject to boundary restraint. In the particular example under consideration, radial and circumferential strains are induced on the cross-cut face. Incidentally, it follows that similar strains, of gradually decreasing amount, are induced at all sections across the tree beyond the cross-cut face, up to the limit of length over which primary longitudinal strain is partially relieved.

It is now possible to derive an approximate mathematical expression of the forces involved. Equation (1) developed from Jacobs's work will be adopted to represent the longitudinal strain at any point in a tree cross section. If e_y represents this strain at any radius r in a standing tree, then

$$e_y = -0.02555 (r_0^{0.075} - r^{0.075}) + t_0.$$

Referring to the cross-cut log illustrated in Figure 4, if l represents the distance beyond the cross-cut face over which some release of strain occurs, the total longitudinal displacement of any point beyond the plane of the original face will be some function of l , say $f(l)$, multiplied by the strain at the particular radial distance from the pith. Thus, if y represents the longitudinal distortion at the face and at distance r from the pith,

$$y = f(l) [-0.02555 (r_0^{0.075} - r^{0.075}) + t_0]. \quad . \quad . \quad . \quad (6)$$

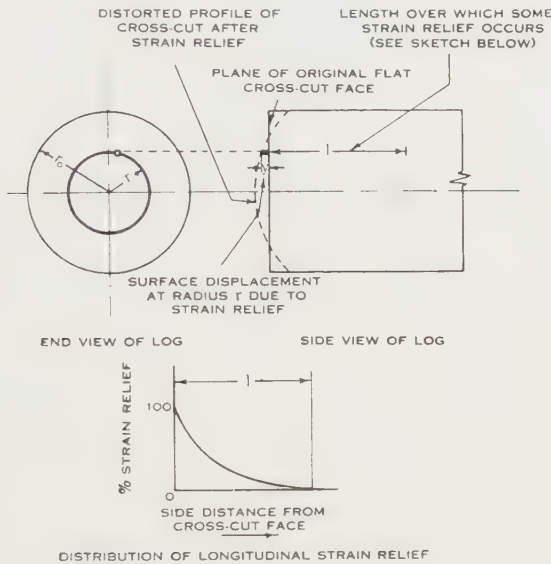


Fig. 4.—Longitudinal strain distortion of a cross-cut face.

This equation represents the movement of a point on the curved surface developed from the originally flat cross-cut surface. The slope of this surface at a particular position on a radial plane is represented by dy/dr . If, to simplify the illustration, $f(l)$ is treated as independent of r , then

$$\frac{dy}{dr} = \frac{0.001916 f(l)}{r^{0.925}}.$$

Also, the radial strain (due to curvature) at any point on the distorted cross-cut face

$$\begin{aligned} &= \frac{\text{length of the curved face} - \text{its original (flat) length}}{\text{original length}} \\ &= \frac{ds - dr}{dr}, \end{aligned}$$

when infinitely small lengths are considered. But

$$\frac{ds}{dr} = \pm \sqrt{1 + \left(\frac{dy}{dr}\right)^2}$$

$$\begin{aligned}
 &= \pm \sqrt{1 + \left[\frac{0.001916}{r^{0.925}} f(l) \right]^2} \\
 &= \pm \sqrt{1 + \frac{0.00000367}{r^{1.85}} [f(l)]^2}.
 \end{aligned}$$

If the radial strain at radius r is denoted by e_r ,

$$\begin{aligned}
 e_r &= \frac{ds - dr}{dr} \\
 &= \sqrt{1 + \frac{0.00000367}{r^{1.85}} [f(l)]^2} - 1. \quad \dots \dots (7)
 \end{aligned}$$

Thus, in any particular case in which $f(l)$ is known, it is possible to determine the strain distribution on the log cross-cut face, arising from strain energy release. On this simplified basis, and using data from the experiments described, it may be deduced that strains having an equivalent radial tension value of the order of several thousand lb./sq. in. tend to be developed near the pith of trees of some species. The strain may fall away rapidly, however, and reach values of no practical significance beyond the central few inches of tree diameter.

The nature of failure in heart shakes—a failure due to excessive ring stresses towards the centre of the tree—indicates that ring stresses are usually more critical than radial stresses. It can be shown that the ring stress intensity arising from the strain energy effect does not decrease as rapidly as the radial stress with increasing distance from the pith. Theoretically, these stresses may be calculated from the radial strain.

Under the conditions in which the release of longitudinal strain energy causes distortion of a cross-cut face, the total change of length along any radius of length r from the pith

$$= \int e_r dr,$$

and the mean strain

$$= \frac{l}{r} \int e_r dr.$$

But the circumferential strain at radius r is equal to the mean radial strain. Hence,

$$\text{ring stress at radius } r = \frac{E_T}{r} \int e_r dr.$$

Or substituting the value of e_r from equation (7),

$$\text{ring stress at radius } r = \frac{E_T}{r} \left\{ 1 + \frac{0.00000367}{r^{1.85}} [f(l)]^2 \right\}^{\frac{1}{2}} dr - E_T.$$

Even with the approximations already adopted, a solution of this expression is complex. Substitution of more precise values for $f(l)$ and its relation to r would complicate the calculations further. At this stage, results available are insufficient to justify evaluation or presentation of the more exact analysis.



Longitudinal strain measurements.

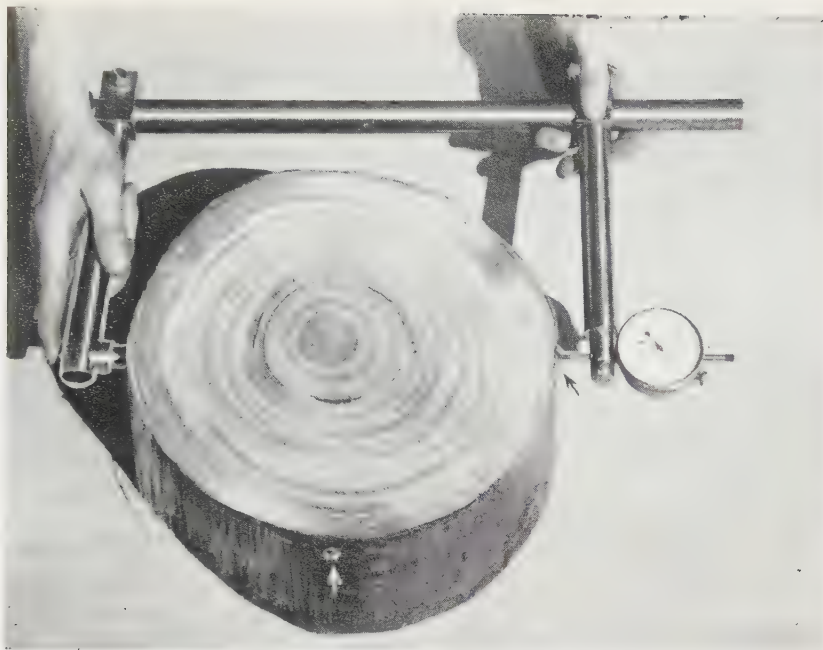
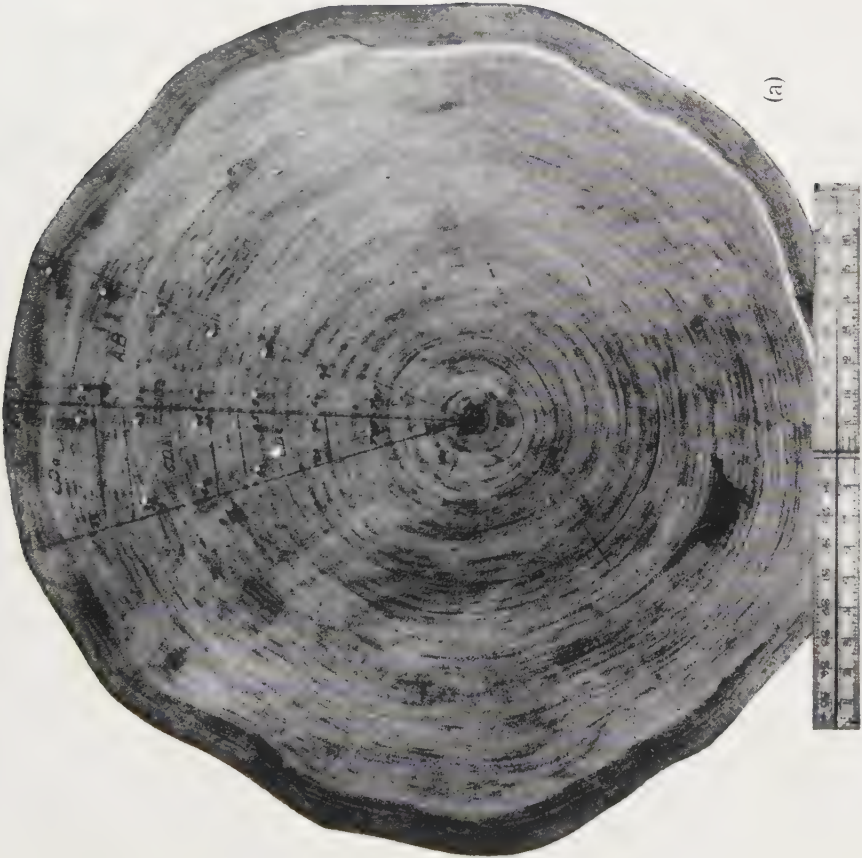


Fig. 1.—Measurement of transverse strain. Diameter gauge on tree section.
Note reference nail under dial gauge and at lower centre.



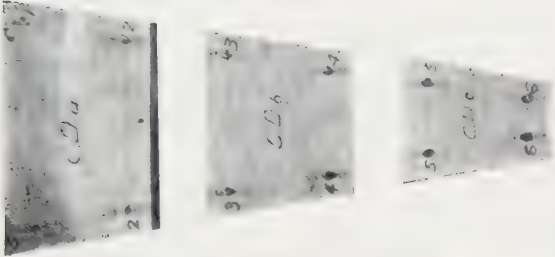
Fig. 2.—Measurement of transverse strain. Typical discs, showing cores cut out and each annulus ready for diameter change measurement.



MARKED DISC

Radial strain measurement.

(a)



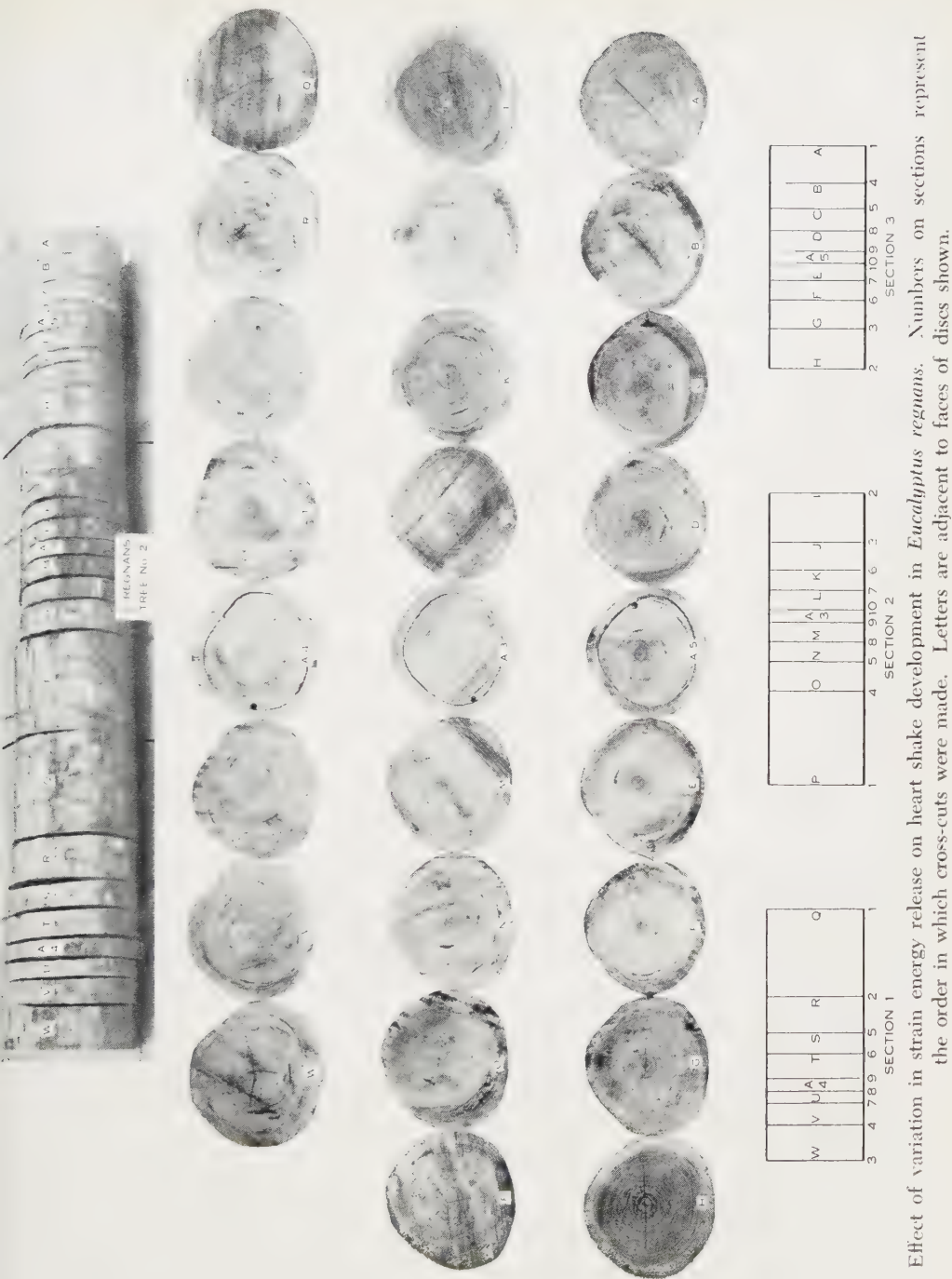
(c)

WEDGE SUBDIVIDED



(b)

WEDGE CUT FROM DISCS



Effect of variation in strain energy release on heart shake development in *Eucalyptus regnans*. Numbers on sections represent the order in which cross-cuts were made. Letters are adjacent to faces of discs shown.

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TREE GROWTH STRESSES

III. THE ORIGIN OF GROWTH STRESSES

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Summary

Evidence available indicates that tree growth stresses originate in newly differentiated cells towards the periphery of a tree.

It is considered that an origin connected with the structural development of peripheral cells would explain both the longitudinal and lateral distribution of growth stress effects more satisfactorily than other hypotheses suggested.

The cause of growth stress development may be explained by the theory that cells tend to shorten in length, and simultaneously to increase in cross section during or immediately after differentiation. The resistance of the mature cells throughout the tree to such a shortening could induce the stress effects which occur.

I. INTRODUCTION

A number of observers, including Martley (1928) and Koehler (1933), have noted reactions of sawn planks, logs, and trees which suggested that significant stresses existed in directions respectively parallel and transverse to the height of growing trees. Jacobs (1938, 1939, 1945) not only confirmed the existence of these stresses in trees, but his many measurements indicated their magnitude and distribution. Clarke (1939) reviewed the information then available. His discussion referred to results to be expected in trees under the conditions of normal growth, and also to features associated with the development of tension wood and compression wood. Additional information and development of data on stresses in trees is now available (Boyd 1950a).

It is concluded that these manifestations of stress are related in some way to the growth conditions of the tree, but from this point proposed hypotheses on the origin of growth stresses are frequently inadequate to explain observed effects. Stress distributions indicated by the induced strains measured by Jacobs (1938, 1945) suggested to him that both longitudinal and transverse stresses have an origin close to the periphery of the tree. This conclusion arose from the fact that strains measured were of uniform magnitude at the periphery, throughout the wide range of diameter classes investigated. Though most measurements were made on alpine ash (*Eucalyptus gigantea* Hook. f.) similar results were obtained for a wide range of species. At the same time, it was shown that the tendency to develop and increase longitudinal compressive strains in the area towards the pith was a function of tree diameter—a result which might be anticipated if the causative stress generation occurred at the periphery and always tended towards an approximately uniform value.

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Though Jacobs's data (1938, 1945) on stresses existing in a plane transverse to the length or height of a tree are scanty and conflicting, these and subsequent data and consideration (Boyd 1950a) point to a relatively constant peripheral value and origin of these stresses. The peripheral origin could explain the distribution of both ring and radial stresses (Boyd 1950a).

Hypotheses on stress generation have been proposed by Martley (1928), Koehler (1933), Münch (1938), Clarke (1939), and Jacobs (1945). However, lack of compatibility of the proposed hypotheses appears to warrant their critical examination.

II. LIMITATIONS OF EXISTING HYPOTHESES

Some hypotheses of stress generation within trees have been based on inadequate data. Frequently, theories were proposed to explain particular features such as longitudinal stress reactions, transverse stress reactions, or the effect of special tissue, and little attempt has been made to correlate all such manifestations in one general explanation. Hence, besides examining the adequacy of the theories within the particular limitations proposed, it is felt that this wider basis of appraisal may justify consideration.

(a) *The Effect of Tree Weight*

After observing that a nominally flat end of a beam became curved in profile after cross-cutting, Martley (1928) reasoned that this was caused by stresses naturally present in the timber. He proceeded to calculate the stress which might develop from tree weight on the basal section of trees of various sizes and species, and concluded that values up to 200 or 300 lb./sq. in. were the maximum which could be expected. On the other hand, he estimated that the observed elongations towards the centre of a cross-cut face must have been the result of stresses of a considerably higher magnitude. Subsequent investigations (Boyd 1950a) have shown that the observed elongations are directly related to longitudinal strain energy movements in the log, rather than to the longitudinal stress alone, but measurements of this stress in hardwoods have indicated values of approximately 3,000 lb./sq. in. (Jacobs 1945; Boyd 1950a).

In addition, the longitudinal strain pattern established (Jacobs 1945; Boyd 1950a) indicates that an annulus or zone of tension exists round the full periphery of the tree—a condition incompatible with the weight effect arising from either symmetrical or asymmetrical growth of the crown. Further, the peripheral tension is approximately constant both round any particular section, and over the height of the tree (Jacobs 1945). On the other hand, tree weight would be expected to cause either compressive stresses only on a tree cross section, or an unsymmetrical distribution of tensile and compressive stresses. Variation of stress with height could also be anticipated if weight were the cause. In pendant branches of eucalypts, it might be anticipated that tensile stresses would predominate if weight were the most significant factor, but actually these branches exhibit a stress distribution similar to that of the bole of the tree (Jacobs 1945).

In a transverse plane, it might be anticipated that tree weight would have some effect. Considering a hardwood, and assuming the normal elastic relationship between primary stresses in a longitudinal direction and induced reactions in a transverse direction, then if the values of the modulus of elasticity in the longitudinal and transverse directions are 3,000,000 and 100,000 lb./sq. in. respectively, and the corresponding Poisson's ratio is 0.45, it can be shown that a longitudinal compressive stress of 300 lb./sq. in. may induce a tensile stress of 4.5 lb./sq. in. in the transverse plane. Although the elastic moduli of a tree may vary appreciably from those assumed above, the order of value of the calculated figure, and the stress character (tensile) appear to be incompatible with measured peripheral, transverse, compressive stresses of the order of magnitude of 300 lb./sq. in. (Jacobs 1945; Boyd 1950a).

Undoubtedly its own weight has some effect in producing stresses within a tree, but the estimated value of these stresses, their character, and distribution appear inadequate to explain the stress equivalents of measured strains. Obviously then, the main cause of stress development must be sought elsewhere.

(b) Koehler's Theories on Stress Generation

Koehler (1933) was concerned with stresses which developed in a transverse plane and caused "rift cracks" or shakes across a cross-cut face. He suggested three possible explanations of the observed stress effect—greater circumferential than radial growth, reduction in the turgidity of the older tissues, and chemical changes of the older wood.

Normally, the wood cells developed within a tree are oriented in a direction parallel to the stem or branch (except the ray cells) and are arranged in any cross section so as to form radial lines from the pith. Consequently, as the tree grows, the increasing girth requires a change in the expanse of the new sheaths of cells, and therefore some variation in the number or shape of cells. From time to time it is necessary that new radial lines of cells be developed. Koehler envisaged that this mechanism of growth involved squeezing or possibly stretching of cells to fill the available space, and that this adjustment might be responsible for the stresses developed.

However, it appears that all substantial adjustments in shape that are necessary would occur in the newly differentiated tissue prior to development of the secondary walls. On this assumption, the secondary walls of the cells would be built up with the primary walls already suitably disposed in position and shape to meet the requirements of the growing tree. Because of its great thickness and strength compared with the primary wall (Preston 1947), the secondary wall must be considered as the most significant in growth stress considerations. Consequently, as this secondary wall is probably not subject to external stresses due to the non-compatibility of uniform cell size and radial and circumferential growth, the corresponding stress effect on the complete cell is likely to be small. Consideration of the elasticity of the wood structure will indicate that in the longitudinal direction, any induced stresses would be much smaller still. Doubts in the validity of this theory of stress development

are increased when it is proposed that some sheaths of cells are squeezed while others are stretched, or at least squeezed to a varying degree. Such an arrangement may render it difficult to explain the relatively uniform strain pattern observed as the tree grows (Jacobs 1945).

That the reduction in turgidity of the older tissue within a tree cross section may cause the development of radial tension and circumferential compression has been proposed (Koehler 1933). Although, in some species, certain cells may retain their turgidity for a considerable number of years, in most species the small proportion of living cells in the sapwood suggests that the effect of their turgidity in inducing transverse stresses throughout the whole cross section of the tree is probably small. Furthermore, after the formation of heartwood has commenced, there is a tendency for the volume of sapwood in proportion to the total tissue in a tree cross section to decrease as the tree grows in diameter (Trendelenburg 1939). To a corresponding degree it might be anticipated that the effect of the turgid tissue in stressing the tree would decrease, and that in a relatively old tree the effect would be small compared with that in a younger tree. Though in some cases immature trees develop severe heart shakes and therefore show evidence of appreciable stresses, this effect is by no means confined to small trees of the particular species (Boyd 1950*b*). In addition, the peripheral transverse stress development has been shown to be approximately uniform in a variety of tree diameters and species (Boyd 1950*a*) even though some of the logs investigated were entirely of sapwood. The evidence therefore appears to be at variance with the hypothesis proposed.

Koehler suggested an alternative hypothesis that chemical shrinkage of cells subsequent to their death might be responsible for the observed indication of stress. If this were so, arguments similar to those above would apply, except that the stress effect of chemical shrinkage, though at first negligible, would gradually become of ever-increasing importance. Again this is not in accordance with observed effects and experimental results.

A combination of the effects of turgidity and chemical changes might be argued as a possibility. However, this, too, would leave unexplained the observed stress development in very young trees (Jacobs 1945).

(c) The Effect of Sap Stream Tension

In view of the considerable amount of investigation of growth stresses by Jacobs, and the facts that, after considering a wide variety of possible causes he stated (1945) that "present evidence points to sap stream tension being the cause of the longitudinal stresses of erect hardwood stems and larger coniferous stems," and that it may be responsible for the lateral stresses also, detailed analyses may be justified.

Many physiologists have examined the phenomenon of sap ascent within a tree, and various hypotheses have been suggested. The different theories proposed imply different sap pressure or suction distributions with correspondingly different stress effects within the tree. However, the hypothesis most

generally held (Thomas 1937) is that the active transpiration of the leaves causes suctions of sufficient strength to draw water to the top of the highest trees. Dixon (1924) has shown that the sap stream could sustain the necessary tension to achieve this end, but in the matter of how the suction is generated, and also the intensity of forces involved, considerable difference of opinion exists. Beyond assuming that the motive force is created in the region of the leaves, it is not proposed to consider the mechanism of sap ascent further. However, the intensity of forces generated will be discussed.

Suction intensities ranging from 10 to hundreds of atmospheres have been estimated by different workers. Thomas (1937) states that suctions equivalent to 10-20 atmospheres are not uncommon in the leaves of trees, and that the forces of adhesion between water and cell wall materials may be over 1,000 atmospheres. Dixon's work (1924) indicates that for some plants, the frictional resistance to flow through the conducting passages may require that the suction exerted be equivalent to twice their height. Obviously this will vary with the particular tree or species, but if one were 340 ft. high, a suction magnitude of 20 atmospheres may be anticipated.

The manner in which sap tension might transfer stresses to the cell wall probably depends upon the mechanism of sap movement, diameter and continuity of the sap columns or conducting passages, rate of transpiration, etc. However, if peripheral longitudinal tensions of the magnitude of 2,000 lb./sq. in. (Boyd 1950a) in the mature wood formed over a period of a few years are to be explained in this way, the average suction exerted in the sap stream over this period must have been very large. Even if the suction developed at the leaves could be transferred as equivalent stress to the cell walls—an unlikely possibility—then a suction intensity of at least 140 atmospheres would be indicated. Taking account of variation of transpiration rate with temperature, relative humidity, wind strength, and seasonal conditions, a considerably higher peak suction intensity may be required to account for the measured stresses in the wood. If such sap stream suction intensities were produced, evidence may be available from other considerations of tree growth. Some possible implications will be examined.

(i) *The Effect of Suction on Cell Walls.*—Little information is available, but on the basis of published cell sizes and wall thicknesses, and on the assumption that on the cambium side the immature cells add little to the strength of adjacent cells, and that in such a species as alpine ash the nominal ultimate strength of wood is only approximately one-third of the stress within the cell wall at failure (when allowance is made for the cellular nature of wood), then a calculation of strength may be made. The internal suction intensities which might be required to produce flexure failures in the walls of vessels, early wood, and late wood fibres are estimated at 30, 150, and 1,500 atmospheres respectively. If these cells are subject to suction forces of the magnitude of 140 atmospheres and perhaps considerably more (which appears necessary if sap tension is to account for measured longitudinal stress development in the wood), then the possibility of fracture or collapse of vessels and early wood fibres must be very real.

The absence in published literature of evidence of inward collapse of green tissue of growing trees therefore raises doubts as to whether the supposed high-intensity suctions are developed, and whether sap stream suction intensities could be responsible for the tree growth stresses measured.

If the calculations indicated above are not even approximately correct (the problem is a complex one), at least it would appear that the supposedly large suction forces may give rise to considerable stresses, and have an appreciable effect on the reactions and characteristics of the wood tissue. A little thought on the hydraulic factors associated with sap flow suggests that if a suction of 140 atmospheres or more were created within the leaves, this large flow potential must be dissipated in overcoming the resistance to sap movement through the small channels and past the many obstructions en route. For this reason, and because of the static pressure difference due to the height of the tree, only a fraction of the suction effect would exist towards the base of the tree. In fact, Tiemann (1937) reported that suctions of appreciably less than one atmosphere have been measured by pressure gauges attached to the base of a tree and connected to the sapwood. Therefore, if the hypothesis of very large suction intensities in the leaves is valid, from the considerations above it would appear likely that a vastly different internal stress existed within conducting wood tissue and any connected tissue near the top of the tree, as compared with similar tissue at its base.

In reference to the cross-sectional shape of cells at different heights within a tree, no very obvious differences have been reported which would suggest that the cells near the top have been subjected to extremely high suction stresses, while those near the base were free of that effect. The absence of reported differences casts doubt on the sap stream theory as the major origin of growth stress development.

(ii) *Lack of Variation in Peripheral Longitudinal Strain with Variation in Size of Tree.*—In discussing the mechanics of sap flow, Thomas (1937) suggested that the suction necessary depends directly upon the length and size of the conducting channels and the height of the tree. If this is approximately correct, then when a sapling 20 ft. high is compared with a tree 200 ft. high, it might be expected that the sapling would require to develop at its leaves only a fraction of the suction required at the leaves of the large tree. The possibility that the leaves of both trees may be capable of inducing the same suction intensity is not questioned, but the continuous generation of greater sap tension than would satisfy the sap flow needs of the tree appears unlikely. On the other hand, it is possible that any tendency to do so may be immediately counterbalanced by the resulting surfeit of sap flow. Although it is not known if differences in sap stream suction have been investigated in two such examples, on the hypothesis that growth stresses result from the sap stream tension it may be logical to expect significantly different growth stress intensities in the two tree sizes quoted.

However, measurements of the longitudinal tensile stress at the periphery indicate that the same growth stress is developed in small saplings and large trees (Jacobs 1938). This casts additional doubt on the sap tension growth stress theory.

(iii) *Lack of Variation of Longitudinal Stress with Variation in Height in the Tree.*—As a corollary of the comparison of likely sap stream suction differences in small and large trees, it is possible that appreciable variation occurs with change in height within a single tree. Again, if the intensity of sap tension is an important factor in the development of growth stresses, then considerable stress variation with change of height in the tree might be anticipated. No such wide variation of peripheral longitudinal stress has been measured. On the contrary, Jacobs (1938) reported an approximately constant longitudinal stress in the sapwood at different heights.

(iv) *Lack of Variation of Peripheral Circumferential Stress with Diameter and Height Variation.*—It has been shown that peripheral longitudinal stresses are approximately constant with variation of tree size (Jacobs 1945). Peripheral circumferential stresses have been similarly demonstrated as uniform (Jacobs 1945; Boyd 1950a). It appears that these are either dependent upon one another—a condition shown to be unlikely (Boyd 1950a)—or they are both produced by some other agency. If the sap stream tension is visualized as that agency, then its possible wide variation with tree height does not appear compatible with the relative constancy of peripheral circumferential stress with both diameter changes in different stems and height variations within a particular stem.

(v) *The Relationship of Sap Stream Forces and Measured Longitudinal Stresses.*—It is desirable to check whether the stresses which might be anticipated from the effect of sap stream forces are of the same character (tensile or compressive) as those measured in the tree. Jacobs (1945) suggested that “the contraction measured when an outer strip is cut from a plank would represent an adjustment of the dimensions of the woody elements of the strip as a reaction to the tension remaining in the sap columns. In the stem itself, the sap tension of each new outer sheath of cells would compress the cross section as a whole.” Thus, with this theory, he envisages that the measured contraction in length of wood at the periphery actually represents a compression imposed as the sap columns are cut across, and not, as he suggests elsewhere (1938, 1945), a tension which is relieved when the piece is freed of the restraint of the tree. He says the sap columns “will contract until the capillary force exerted at the apertures in the pit membranes, or other obstructions in the lumina of the cells, balances the tension remaining in the columns. The remaining tension will then be held, and a longitudinal compressive reaction will be imposed on the woody substance.” The increase of length of wood from the core is explained as a reaction to the compressions developed over a period by the natural breaking of sap columns within the tree.

This hypothesis implies that when the sap columns are continuous in the growing tree, they do not impose significant stresses on the tissue through which they pass, but develop stresses in adjacent tissue as a result of a tendency to suck out the fluid contained in its broken sap columns. However, when a tree has been felled the continuity of all columns between roots and crown has been broken, and therefore there must be a tendency to develop the full tension within these as discussed above. Presumably the sap tension developed under such circumstances would depend largely on the tendency to dry out or remove moisture from the columns. With vessels within the wood, the removal of fluid may be comparatively easy, but because of the relatively large diameters of cells concerned, the sap tensions developed would be equivalent to a capillary rise of not more than a few feet (Thomas 1937) and the corresponding stresses must be insignificant in relation to the measured stresses in trees. With wood fibres, the removal of fluid corresponding to the development of stresses would probably be considerably slower. Yet, as a tree is cross-cut under experimental conditions (Boyd 1950*a*), all observable stress reactions, both in the sapwood and towards the pith, occur immediately after cutting, and no significant addition to the stress development occurs over a period of hours in ordinary atmospheric conditions. In Jacobs's experiments (1945) also, no effect due to the probable wide range of atmospheric conditions during measurement was noted.

If the initial cross-cutting of a log severs the sap columns, on this theory the corresponding tension must soon be established in them, and a compression imposed on the wood. Then additional cross-cutting should not affect either the general intensity of sap tension or the imposed longitudinal compression in the wood. In fact, in Jacobs's experiment (1938, 1939, 1945), strips 8 ft. long reacted to the full stress development of uniform intensity along their entire length. However, if strain gauges are placed on the sapwood at a position between the ends of a log, it can be shown (Boyd 1950*a*) that reduction of length equivalent to the full measured peripheral stresses (Jacobs 1945) occurs as a result of additional cross-cutting. It appears, therefore, that the longitudinal stress in the sapwood of a tree is not caused by the breaking of sap columns, such as results from felling or cross-cutting, but rather that cross-cutting or stripping the tree into beams may release stresses previously present in the tree. In the sapwood the stresses released are tensile, but the sap tension theory indicates compressive stresses.

Jacobs (1945) has shown that a progressive compression is imposed on the wood towards the pith. This may be difficult to explain in terms of sap tension. When the sap columns break, the compressive forces supposedly imposed by them upon the wood towards the pith are relieved. The development of new sap columns may reimpose the same force, but a cumulative stressing effect is doubtful. Actually, Trendelenburg (1939) shows that the proportion of sapwood tends to decrease as the tree matures, and therefore the effect of forces applied through the sap stream to the heartwood may conceivably decrease. Thus, instead of a cumulative compression of the core, a reduction of compression may occur.

Consequently, from the many aspects considered it is doubtful if growth stresses could be explained in terms of sap stream forces.

(d) *The Effect of Vacuolation Forces*

A vacuolated living plant cell may be regarded as an osmotic system (Thomas 1937). The pressures generated as a result of osmosis may account for growth stress development within a tree. Jacobs (1945) states that "the pressure of vacuolation must be great enough to rupture the bark and enable a tree to accomplish diameter growth," and he suggests that transverse growth stresses may be explained in this way.

In regard to the pressure required for splitting the bark, it is conceivable that a few pounds per square inch would suffice. For example, if a radial pressure of 5 lb./sq. in. were exerted on the inside face of the bark of a 10 in. diam. tree, the splitting force per inch of length (vertical) of bark considered would amount to 25 lb. This force would tend to increase with diameter increase and undoubtedly a force of this order of magnitude could account for splitting the bark of many species. However, it has been shown (Boyd 1950a) that a radially directed pressure intensity R at the periphery induces a peripheral circumferential stress equal to $R \times E_T/E_R$, where E_T and E_R represent the elastic moduli for forces applied in the tangential and radial directions respectively. Measurements made in this laboratory on several green specimens indicate that the ratio E_T/E_R approximates 0.5 for *Eucalyptus regnans* and 0.6 for *Pinus radiata*. Similar results for a variety of species may be derived from published data of the U.S. Forest Products Laboratory, Madison, Wis. (Report Series No. 1528, 1945-6). Thus the peripheral circumferential stress equivalent to a radial pressure of 5 lb./sq. in. would be of the order of 3 lb./sq. in. This is considerably less than measured peripheral transverse stresses in a wide range of species which range from a "probable minimum of 30 lb./sq. in. to a probable maximum of 360 lb./sq. in." (Jacobs 1945). Consequently it is considered that forces much greater than necessary to split the bark are probably operating.

In an attempt to determine vacuolation pressures, Jacobs (1945) applied pressure pads to growing trees of *Pinus radiata*. He found that circumferential growth continued in the local area under a pad of 1 sq. in. area and loaded with a force of 150 lb. From this he concluded that growth could, and normally probably did occur with pressures of vacuolation approximating 150 lb./sq. in. (10 atmospheres).

However, reservation should be placed on conclusions drawn from this test. In a general way, it is felt that the knowledge that a tree can generate sufficient force under stimulus to continue circumferential growth against restraint may be an insecure basis for concluding that a like force is constantly or normally generated without that particular stimulus. This doubt is reinforced by Jacobs's own observations and photomicrograph (1945), which indicate that, when the tree was subjected to a pressure on the load pad of 100-150 lb., the shape of cells formed was significantly different from normal.

Jacobs assumed that the pressure equivalent of a pad load of 150 lb. was 150 lb./sq. in. However, this intensity may be much greater than the actual pressure intensity under the pads. As the square loading pads were applied to the outside of the bark, its stiffness would tend to support the load on all four sides of each pad. Very little side support beyond the edges of the pad would cause the effective area of application of the load to be considerably increased, and a spread of less than $\frac{1}{4}$ in. on all sides would reduce the intensity of the 150 lb. load application to 75 lb./sq. in. To allow of checking this point, Dr. Jacobs kindly made available the load pad area of one of his specimen trees. A radial saw cut was made through the centre of a load area and a photograph of the exposed face is shown in Plate 1. It may be seen that the grain of the wood towards the bottom of the photograph is straight. This wood was formed before the pad was applied. The curvature of the resin ducts in the wood formed after application of the pad indicates the general disposition of tissue, and confirms the fact that the applied pressure is distributed for an appreciable distance beyond the edge of the 1 in. square pad. A cut made in the transverse direction showed a similar spread of the pressure effect.

In addition, Jacobs states that an applied force of 25 lb. had a positive effect in restricting diameter growth. Again allowing for pressure distribution, it appears that relatively very low pressure intensities may restrict the effect of vacuolation forces. For this to be so, probably the vacuolation forces themselves are correspondingly small under normal growth conditions and consequently they appear inadequate to explain transverse growth stresses.

Vacuolation forces will have a direct effect in the longitudinal direction of the cells and the trees. However, by comparison with stresses in a closed cylinder, and on the assumption of approximate cell dimensions, it may be estimated that a vacuolation pressure of 10 atmospheres would develop a longitudinal stress in the cells of 100-200 lb./sq. in. As these calculations are based on vacuolation stresses which may be high, and the values obtained are very much below measured peripheral longitudinal stresses of 1,000-2,000 lb./sq. in. (Boyd 1950a), the vacuolation pressure origin does not provide a satisfactory explanation of longitudinal stresses. Also, adopting approximate values of the relation of induced longitudinal strain resulting from transverse strain (Kollmann 1936) it may be estimated that, even if vacuolation pressures of 10 atmospheres or more were developed, these would induce stresses probably very much less than 100 lb./sq. in. in the longitudinal direction, and either alone, or in combination with the direct longitudinal effect of these forces, could not explain the origin of longitudinal growth stresses. Furthermore, as vacuolation pressure or the effect of turgidity of cells diminishes and possibly disappears with the completion of the cell wall of structural wood tissue, its effect in stressing the wood is apparently not cumulative. On the contrary, as the trees grow the stress effect would tend to become smaller and could not explain the actual stress distributions observed.

(e) Development of Stress in Compression Wood

It is generally acknowledged that the characteristics of compression wood are very variable, and even in the one tree the most extreme type of abnormal tissue is associated with other tissue whose properties gradually merge into those of normal wood (Pillow and Luxford 1937). Münch (1938) considered some of the possible mechanisms by which a leaning tree might tend to grow straight under the influence of compression wood. He postulated that the corrective force arose from a positive longitudinal growth of compression wood cells as they matured, and a resistance offered by adjacent tissue to that growth. In this way he envisaged the generation of a dynamic stress in the wood structure.

Brown (1944) stated that the "secondary wall is not formed until the enlargement entailing sliding growth is practically complete." Also, Frey-Wyssling (1948) stated that the secondary walls are "deposited by apposition against the fully grown primary wall during the corresponding growth of the membrane in thickness." During growth, it is probable that the primary wall, because of its extremely fragile character (Preston 1947), could not impose large forces on the mature wood tissue. Hence there is a definite doubt that the necessary stresses required for straightening a tree of a coniferous species have their origin in a positive lengthening of the relevant wood tissue.

III. CELL WALL SHORTENING AS AN ORIGIN OF TREE GROWTH STRESSES

Münch (1938) suggested that the extremely large longitudinal stresses presumed to be associated with tension wood arise from a tendency of these cells to increase in cross section and simultaneously to reduce their length. The abnormal stress development of this reaction wood is confirmed by Jacobs (1938). Clarke (1939) remarked that the same tendency to shorten and broaden the cells may well be present in normal wood and account for the transverse compressive stress mentioned by Koehler (1933). Though ultimately Jacobs (1945) preferred the sap stream hypothesis he initially suggested that "the most reasonable theory for the cause of fibre tension on present knowledge would be that as the wood elements solidify from the gelatinous cambium they tend to shrink slightly, the tendency being resisted by their adhesion to the inner core." As no known attempt has been made to develop these hypotheses in conjunction with experimental data, it is proposed to test them in the light of information available.

Apart from the actual mechanism involved, Münch's suggestion of a simultaneous shortening and dilation of the maturing cells, if applicable to normal wood, may explain the simultaneous peripheral development of longitudinal tension and ring compression. A notable feature of this stress arrangement is the approximately constant intensity of stress generation in both directions at all stages during growth.

(a) Strain Capacity of Wood Cells

As a convenient method of investigation of tree growth stresses, Jacobs measured the normally associated longitudinal strain or proportional change in length of measured sections under the influence of growth forces. These strain determinations were made on strips of sapwood of varying radial thickness ranging up to $\frac{1}{2}$ in. (which was generally adopted for trees 6 in. diam. and greater).

A study of Jacobs's measurements, and particularly the graph prepared by him (1945) to show the radius of the tree plotted against the unit contraction (strain) rate of the core under the influence of the longitudinal strain developed in the sapwood, reveals that the wood in the sapwood zone develops remarkably different strains in trees of different radii. At the same time, it must be pointed out that the strain recovery of the sapwood when released from the restraint of the tree is approximately constant over the wide range of diameter classes measured (from 0.1 to 30 in.). In alpine ash, the mean recoverable sapwood strain (tensile) was 0.0008, and a similar figure was obtained in many other species of pored timber. The tensile stress equivalent of this strain in alpine ash is 1,200 lb./sq. in. (Jacobs 1945).

A conclusion which may be drawn from the graph is that, for the diameter classes $\frac{1}{4}$ in., $\frac{1}{2}$ in., 1 in., 2 in., 5 in., and 20 in., the respective strains imposed by the peripheral tissue on the whole enclosed core are 16, 8, 5, $2\frac{1}{2}$, 1, and $\frac{1}{4}$ times the residual or measurable strain at the periphery. Since the residual peripheral strain is constant with variation of height within the tree, it may be expected that individual cells would have mean strains equivalent to that measured in the wood of which they form part. In this range of diameter classes then, not only do peripheral cells exhibit residual strains of 0.0008, but also they impose strains on the cells enclosed by the peripheral sheaths ranging in ratio from 64 to 1, and equivalent to as much as 16 times the measured residual peripheral strains. This implies that a change in length of peripheral cells occurs during the process of stress development, resulting in a maximum shortening during maturity in peripheral cells differentiated while the diameter is small, and progressive proportional change in peripheral tissue with increasing diameter.

Since the changes in peripheral cell length are always greater than, and range up to many times the maximum recoverable strain, it may be deduced that at the stage of stress development, or over the limited period after cell formation represented by the width of sapwood strip examined, a tensile strain of 0.0008 (approximately) is the maximum which can be elastically sustained within the cells. Therefore, other changes of length must be accomplished under conditions of plastic strain or irrecoverable change of length of the cell walls. If then the structural characteristics of the cell walls can generally be regarded as similar over most of the tree diameter, the forces successively developed in the peripheral tissue as the tree grows must be such as would tend to shorten these cells in conformity with the greater measured shortening associated with cells developed towards the pith. With increasing diameter,

the restraint exerted by the mature core of wood on free reduction of cell length would have the effect of increasing the plastic flow and causing a corresponding permanent strain in the cell walls.

It may be of interest to note here that such a system of strain generation and restraint, if imposed on cells which were originally of uniform length over the range of tree diameters, would result in somewhat longer cells as the tree diameter increased. From the early stages of tree growth to the later stages, the rate of cell length increase would at first be relatively rapid, later a constant length would be approached. However, it should be realized that the extreme change in length of cells in the tree diameter range of $\frac{1}{4}$ in. to 30 in. would approximate only 1/100 of the cell length. Even in the ideal case this would be difficult to measure, but the changes in cell lengths which occur under the various growth stimuli and independently of the stress effect would completely mask it in any practical example.

(b) The Relationship of Growth Stresses and Cell Wall Deformations

Cell wall deformations have been the subject of considerable investigation by a number of workers. It is proposed to discuss them in some detail in a separate paper, but because of their possible bearing on the study of tree growth stresses brief mention will be made below.

Wardrop and Dadswell (1947) have stated that "slip planes occur commonly in nearly all fibres and tracheids with perhaps some exception in the case of the wood in close proximity to the pith of the main stem or branch. They have definitely been observed in the cell walls of the last-formed fibres of many trees, i.e. in the fibres just differentiated from the cambium." The same workers have shown that these distortions have the appearance of a crinkle or fold very much akin to that of a compression failure.

As growth stresses appear to be developed in cells close to the cambium zone, it is perhaps reasonable to consider that cell wall distortions may be connected with that development. However, if growth stresses resulted from sap stream tension, longitudinal tension would be general throughout the cell walls of conducting tissue in the sapwood. This would appear incompatible with the compression-type cell wall distortion. On the other hand, if growth stresses resulted from some mechanism causing shortening of the differentiating cell wall, then newly formed cells would be subject to considerable compressions by younger cells just maturing from the cambium. This compression may be sufficient to cause the distortions.

The distribution in tension wood zones of the more severe cell wall distortions known as "minute compression failures" (Wardrop and Dadswell 1947) may provide additional confirmation. In comparing the reaction of normal wood and tension wood, if it is assumed that the orientation of the cellulose molecular chains is similar in the wall layers common to both types of cells, then the application of a particular force with an origin external to the cell wall might be expected to have a less severe effect in stressing the relatively thick-walled tension wood fibres than would be expected in normal wood.

Yet minute compression failures are more common in tension wood tissues than adjacent normal wood tissue. As it does not appear likely that sap tensions would be relatively greater in tension wood, again the sap tension theory appears incompatible; but if the tensions generated were in some way related to the volume and other characteristics of the material in the maturing cell walls, then exceptionally high stress intensities in tension wood cells might be expected. The reaction of the attached cells on the pith side (whether tension wood or normal wood) may induce compression intensities sufficient to cause severe distortion in them.

(c) *The Mechanism of Growth Stress Development*

The foregoing deductions, which are based on experimental results and observations discussed, are consistent with the idea that the wood cells shorten in length. Generally, this shortening could result either from forces generated external to the cell wall structure or from forces developed within the actual wall; but detailed consideration of possible external agencies such as tree weight, sap tension, etc., have raised considerable doubt that these could explain the observed results. Consequently, the hypothesis which may best fit the facts is that there is a tendency for the cell walls to shorten under the effect of some compelling mechanism within their structure, and that the restraint applied by the relatively rigid mature core of the tree to their free adjustment of length and cross section is responsible for the generation of growth stresses.

In tension wood, Münch (1938) has suggested that, at the time of its formation, the secondary wall undergoes an irreversible swelling, as a result of which it exerts a radial pressure on the outer layers of the wall, tending to compress the spiral windings and shorten the cell. If, as Clarke (1939) suggests, this mechanism in a less extreme form occurs in normal wood, it may well explain the longitudinal and transverse stress distribution which has been observed.

At this point it is desirable to recall that transverse stresses induced as a result of longitudinal stresses alone could not account for the measured values (Boyd 1950a). Consequently, a theory which presupposed shrinkage of the peripheral cells would be unsatisfactory, in that the cross section of cells would be required to increase to account for the measured transverse growth stresses. On the other hand, if increase of cell cross sections occurs either from the effect of fibre swelling or some other cause, this could produce the peripheral circumferential compression required to explain the distribution of radial and ring stresses.

Jacobs (1938) has shown that tension wood is characterized by the development of much greater tensions than normal wood. Also, Wardrop and Dadswell (1948) have shown that tension wood is very similar to normal wood of the same species, except for the addition of a relatively thick unligified tertiary inner wall in the tension wood fibres. This tertiary wall replaces the more normal thin inner layer of the secondary wall. It appears likely then, that the increased volume of cellulose within the cell walls may be directly related to the greater stress development of tension wood tissue.

Münch (1938) has suggested that compression wood achieves the purpose of correcting the lean of a tree by the process of positive cell lengthening. Though this is open to doubt, the same purpose could be achieved if the compression wood fibres shortened to a smaller degree than new tissue on the opposite side of the stem. Compression wood cell shortening may appear to be incompatible with observations of Jacobs (1938), Pillow and Luxford (1937), and others that the compression wood is in a state of compression in the tree, but such observations can be explained by consideration of mechanics quite independent of wood structure. If it is conceded that cell shortening is proportionately less than normal, then it is of interest to note that this occurs in cells which contain proportionately more lignin than normal (Pillow and Luxford 1937).

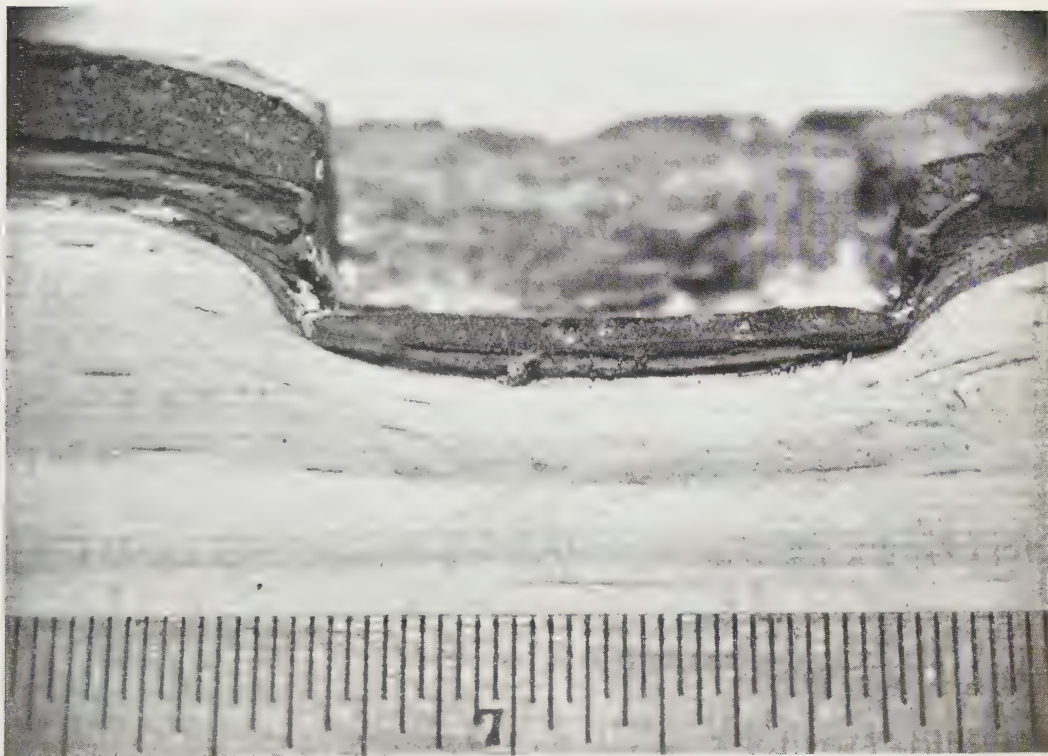
The particular process responsible for the reduction in length of the cells is at present obscure. However, it could conceivably be due to changes in the chemical composition of the cell walls or to changes in the physical state of some or all of the cell wall constituents. It is also possible that lignin, which is normally associated with rigidity in plant tissue (Thomas 1937) may modify the degree to which cells shorten.

IV. CONCLUSIONS

After consideration of various hypotheses, it appears that the cause of tree growth stresses may be found in reactions developing in the differentiating cell walls and causing the cells to shorten and dilate. The reason for the cell shortening (and dilation) is not yet apparent, but consideration of the relative reactions of normal wood, tension wood, and compression wood, and the comparative amounts of cellulose and lignin materials present in each, suggests that the cellulose material may be primarily responsible, though possibly modified in effect by the amount and disposition of the lignin and carbohydrate constituents.

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Disturbance of wood growth and compression of bark under 1 in. square load pad in a specimen of *Pinus radiata*. Applied load was approximately 100 lb. Graduations on scale shown each represent $\frac{1}{32}$ in.

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THE STATUS OF THE TWO SPECIES OF *LUCILIA* (DIPTERA, CALLIPHORIDAE) ATTACKING SHEEP IN AUSTRALIA

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Summary

Evidence presented demonstrates that *L. cuprina* and *L. sericata* are distinct species. A number of characters previously unrecognized are described whereby larvae and both sexes of adults of the two species may be recognized without difficulty. The geographical distribution of the two species is different, as is also their habitat preference and their degree of attraction to living sheep. Furthermore, hybridization experiments indicate that there is great difficulty in obtaining successful matings. *L. cuprina* is the principal Australian sheep blowfly, whereas *L. sericata* is comparatively rare in sheep-raising country and does little damage. There are two subspecies of *L. cuprina*, one from the Oriental and American regions (*L. cuprina cuprina*) and one from the African and Australian regions (*L. cuprina dorsalis*).

I. INTRODUCTION

It was not clearly recognized until 1930 that a species of *Lucilia* was responsible for most of the blowfly strike of sheep in Australia (Mackerras 1930) and until about 1932 the English sheep blowfly *L. sericata* was thought to be the only *Lucilia* species causing strike (Fuller 1932; Mackerras and Fuller 1937), although *L. cuprina* had been recorded from Australia several years earlier (Malloch 1927). Since the early 'thirties a considerable amount of evidence has been accumulated, which demonstrates that the most important sheep blowfly in this country is *L. cuprina* and that this species is quite different in its distribution, habits, and morphology from the economically unimportant *L. sericata*. The distinctiveness of these two species has to a large extent been assumed in most Australian publications dealing with sheep blowfly and no adequate account is available of the differences observed.

For many years in South Africa the Australian practice was followed of regarding *L. cuprina* as the principal sheep blowfly and *L. sericata* as of minor importance. In 1945, however, Ulyett reported that these two species mated in the laboratory and, using colour of fore femora and of abdomen as specific characters, he found that the F_1 hybrid had legs typical of *L. cuprina* and abdomen typical of *L. sericata*. In the F_2 generation these two colour characters segregated in a 1:4:1 ratio. Furthermore, field material always contained individuals having the appearance of hybrids and it was pointed out that, if femur coloration alone was considered, this would lead to all hybrids being considered as *L. cuprina*, resulting in far greater numbers of *Lucilia* specimens

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being recorded as *L. cuprina* than as *L. sericata*. Although it was considered for a time that this conclusion might be valid for the two "species" in South Africa, Ullyett's extension of it to cover the position in Australia was immediately recognized by Australian workers to be unsound. However, since he has recently reaffirmed his belief that the two species are identical (Ullyett 1950) it is important to consider not only the status of these two species in Australia, but also, where possible, their affinities with species bearing the same names in other parts of the world.

II. THE SYSTEMATICS OF *L. CUPRINA* AND *L. SERICATA*

(a) Taxonomy

(i) *The Significance of the Genera Lucilia R-D and Phaenicia R-D.*

Many authors have followed Robineau-Desvoidy (1830) and divided the genus *Lucilia* into two genera or subgenera, *Lucilia* and *Phaenicia*, on the basis of two characters which are illustrated by the following key:

- Subcostal sclerite (at extreme base of stem vein on under side of wing) with short setulose hairs apically. Ocellar triangle reaches half way from the vertex to the lunule in females *Lucilia* Robineau-Desvoidy
- Subcostal sclerite without hairs, although soft pubescence may be present. Ocellar triangle reaches less than half way from the vertex to the lunule in females *Phaenicia* Robineau-Desvoidy

Neither of these characters can be regarded, however, as providing adequate justification for any more than subgeneric status. Both *cuprina* and *sericata* lack hairs on the subcostal sclerite and hence belong to *Phaenicia*. In view of the recommendation of the 7th International Entomological Congress (Rosenbaum 1939) that the use of subgeneric names should be avoided, it is preferable not to use this name and to regard both species as belonging to the genus *Lucilia*.

(ii) *General Remarks on Species*

(1) *Lucilia cuprina* (Wiedemann) 1830.—Originally described as *Musca cuprina* in *Ausserereurop. Zweifl. Insekten*. 2: 654. Type locality: China. Type female in the University Zoological Museum, Copenhagen, and not, as sometimes recorded, in the Leyden Museum (Senior White *et al.* 1940).

Synonyms

Lucilia dorsalis R-D., 1830, *Myodaires*, p. 453. Type locality: Cape of Good Hope. Type male in Paris.

Lucilia amica R-D., 1830, loc. cit. Type locality: Timor. Type male in Paris.

Lucilia elegans R-D., 1830, loc. cit., p. 458. Type locality: Ile de France. Type female in Paris.

Lucilia argyrocephala Macq., 1846, *Mém. Soc. Roy. Agric. Arts Lille*, p. 326; 1846, *Dipt. Exot. Suppl.* 1, p. 198. Type locality: Cape of Good Hope. Type specimen probably in Spinola's collection in Castello di Tassarola (Novi Ligure) (Horn and Kahle 1936).

Musca fucina Walk., 1849, *List. Dipt. Brit. Mus.* 4: 883. Type locality: South Africa. Type in British Museum.

Musca serenissima Walk., 1852, *Ins.* Saunders 4: 340.

Musca temperata Walk., 1852, loc. cit.

Lucilia leucodes Frauent., 1867, *Verh. zool.-bot. Ges. Wien* 17: 453.

Somomyia pallifrons Big., 1877, *Ann. Soc. Ent. Fr.* 7: 257.

Strongyloneura nigricornis Sen. White., 1924, *Spolia Zeylan.* 13: 115. Type locality:

Cherat, North West Frontier Post, India. Type is a male.

Lucilia pallescens Shannon, 1924, *Insec. Inscit. Menst.* 12: 78. Type locality: Wilmington, North Carolina. Type male in U.S. National Museum.

The only species which requires discussion in this list of synonymy is *L. pallescens* (Shannon 1924). Although the author of this species himself later regarded it as synonymous with *L. cuprina* (Shannon 1925, 1926), Hall (1948) lists it as a valid species and gives several characters to distinguish *cuprina* from *pallescens*. These all concern the proportions of various regions of the head, but no definite measurements are given and the differences used (stated in such terms as "not so wide in comparison with . . .", "proportionately higher in comparison with . . ." etc.) do not appear to be valid. A comparison of a large series of *L. pallescens* from Texas, New Orleans, and Washington with Australian *L. cuprina*, using the distinguishing characters mentioned later (Section II (b)), failed to reveal any differences between the two forms.

The authors have come to the conclusion that, over its vast geographical range, *L. cuprina* is represented by two readily distinguishable subspecies. For these the names *L. cuprina cuprina* (Wied.) and *L. cuprina dorsalis* R-D. appear to be most appropriate. Wiedemann's type female *cuprina* has been carefully examined. The general coloration of the fly is dull olive-green (K. R. Norris, unpublished data). The typical subspecies (*L. cuprina cuprina*) occupies a very large area including China* (type locality), Japan*, Formosa*, Assam*, Malaya*, Java*, Soembawa*, Timor, Fiji*†, Hawaii, North America* (= synonym *L. pallescens* Shan.), and South America*. It is easily recognized by its dull olive-bronze body coloration. The brilliant metallic coppery green, green, or bluish green sheen of *L. cuprina dorsalis* is absent or poorly developed. The general coloration of *L. cuprina cuprina* is suggestive of a fly of the genus *Musca* rather than of the genus *Lucilia*. The type specimen of *amica* R-D. from Timor, which is housed in the Paris Museum, is dull (Séguy, personal communication) and belongs to the typical subspecies.

L. cuprina dorsalis R-D. occurs in Africa*, India, and Australia*. It is very widespread in the sheep-raising areas of Australia (see Section IV), and South Africa, it has been recorded from Kenya (Lewis 1933), and the authors have examined one specimen from Cairo. This subspecies is easily recognized by its brilliant metallic coppery green, green, or bluish green coloration, which is similar in general character to that of other well-known *Lucilia* species, such as *L. sericata* and *L. caesar*. No intermediates between this and the other subspecies of *L. cuprina* have been seen.

The name *dorsalis* R-D. has been selected for this subspecies, the type male bearing this name coming from within the geographical distribution of the

* Localities from which material was examined in this study.

† Five specimens, identified by Bezzi (1928) as *L. cuprina*, were available.

bright *cuprina* subspecies (Cape of Good Hope, South Africa, Robineau-Desvoidy 1830). This specimen is housed in the Muséum Nationale d'Histoire Naturelle, Paris. *L. dorsalis* has page priority over *L. elegans* R-D. also from the South African region (Ile de France).

(2) *Lucilia sericata* (Meigen) 1826.—Originally described as *Musca sericata* in *Syst. Besch.* 5: 53.

Type locality: Germany. Type apparently lost. It is not in Paris Museum (Séguy, personal communication), but may be in Halle or Vienna.

Synonyms

Musca nobilis Meig., 1826, *Syst. Besch.* 5: 56. Type locality: Europe. Type apparently lost, but possibly in Paris.

Musca tegularia Wied., 1830, *Aussereurop. Zweifl. Insekten* 2: 655.

Chrysomyia capensis R-D., 1830, *Myodaires*, p. 451. Type locality: Africa. Type in Paris.

Musca pruinosa Meig., 1830, *Syst. Besch.* 7: 294. Type locality: not stated. Type female in Paris.

Lucilia chloris Halid., 1833, *Ent. Mag.* 1: 165.

Lucilia flavipennis Macq., 1842 (*nec Kram.*) *Mém. Soc. Roy. Agric. Arts Lille*, p. 296; 1842, *Dipt. Exot.* 2 (3): 139. Type locality: Ceylon. Type male in Paris.

Lucilia basalis Macq., 1842, *Mém. Soc. Roy. Agric. Arts Lille*, p. 305; 1842, *Dipt. Exot.* 2 (3): 148. Type locality: America. Supposed type in Paris, but bears Mogador as locality.

Musca lagyra Walk., 1849, *List. Dipt. Brit. Mus.* 4: 885. Type locality: Fayal. Type in British Museum.

Lucilia latifrons Schin., 1862, *Fauna Austriaca* 1: 590.

Lucilia sayi Jaenn., 1867, *Abh. senckenb. naturf. Ges.* 6: 375. Type locality: Illinois. Type in British Museum.

Lucilia frontalis Br. and von B., 1891, *Zweiflügler des Kaiserlichen Museums zu Wien* 5: 116, *nomen nudum*. Type locality: Egypt. Type in Vienna.

Lucilia giraulti Towns., 1908, *Smiths. Misc. Coll.* 51: 121. Type locality: Paris, U.S.A. Type male in U.S. National Museum.

Lucilia barberi Towns., 1908, *loc. cit.* 51: 121. Type locality: Williams, Arizona. Type male in U.S. National Museum.

The only additions to synonyms previously published are "species A" and "species B" of Miller (1939). Through the courtesy of Dr. Miller 31 specimens of *Lucilia* "species A" were made available for examination. Most of these were bred specimens of about half normal size, probably caused by inadequate food in the larval stage. Except for coloration, the characters of "species A" are the same as those of *L. sericata*. The lighter coloration of "species A" may be partly due to the fact that the specimens were pinned shortly after emergence and before hardening and darkening had been completed. This is suggested not only by their colour but also by the fact that their relatively light-coloured legs are collapsed and twisted, as often happens when unhardened flies are killed.

"Species B"* is also typical morphologically of *L. sericata*, although its coloration is somewhat lighter. This again may have resulted from the specimen having been pinned before it had hardened and darkened. In Dr. Miller's

* The single "type" specimen was available for examination. This specimen has no locality label and Miller says that it may have been collected in Europe, Australia, or New Zealand. It cannot, therefore, be referred to New Zealand.

collection there are two additional males labelled "species B" which were not mentioned in his 1939 paper. These are two typical male *L. sericata* having normal *sericata* coloration. An examination of many specimens of *L. sericata*

TABLE I
SUMMARY OF SPECIFIC DIFFERENCES BETWEEN *L. cuprina* AND *L. SERICATA* ADULTS
(SEE TEXT FOR MORE PRECISE DATA ON CHARACTERS)

Characters	<i>L. cuprina</i>	<i>L. sericata</i>
Head		
1. Occipital bristles (Figs. 1 and 2)	1 on each side	6 to 8 on each side
2. Clypeus (Figs. 3 and 4)	Black	Light amber
3. Frontal stripe (Figs. 3 and 4)	About as wide as a para-frontal	About twice as wide as a parafrontal
Thorax		
4. (i) Humeral calli (Figs. 5, 6, and 7)	2 to 4 hairs	6 to 8 hairs
4. (ii) Notopleura (Figs. 5, 6, and 7)	3 to 5 hairs	8 to 16 hairs
5. Second pair of presutural acrostichals (Fig. 5)	Do not extend to first pair of postsutural acrostichals	Extend at least as far as insertions of first pair of postsutural acrostichals
6. Scutellar bristles (Fig. 5)	Dorsal bristles slightly smaller than, or equal to, lateral hairs	Dorsal bristles distinctly smaller than lateral hairs
7. Colour of fore femora	Metallic green	Dark metallic blue to black
Abdomen		
8. Hairiness of 2nd, 3rd, and 4th abdominal sternites (only for males)	Hairs longer than on hind femora and tibiae	Hairs about the same length as on hind femora and tibiae
9. Contour of last abdominal tergite	Generally smooth	Collapsed
10. Male genitalia (Figs. 8 and 9)		
(i) Terminal claspers	Long and slender, outer margins of apical third parallel	Broader, tapering regularly, outer margins converge and are not parallel
(ii) Subterminal claspers	Slender, similar and parallel to terminal claspers	Semicircular and much broader than <i>cuprina</i> , particularly at basal half. Widely separated at tip

from New Zealand indicates that they normally have the metallic bluish green coloration typical of this species from other parts of the world and not the "peculiar coppery colour" recorded by Aubertin (1933).

(b) *Distinguishing Features of L. cuprina and L. sericata*(i) *Adults*

In the past, *L. cuprina* and *L. sericata* have generally been separated by differences in coloration of the fore femora (Hardy 1940; Joint Blowfly Committee 1933). Although this is a valuable character, it is not always reliable if the specimens have been killed before they have hardened and darkened properly or if they have been exposed to high temperatures or become fouled by animal or plant juices. The only additional characters available are the differences in male genitalia, readily visible only after dissection, and differences in the hairiness of the male abdomen (Aubertin 1933; Hardy 1940; Malloch 1927).

Ulyett (1945) states that, in *L. cuprina*, the abdomen is green with a bronze sheen, whereas in *L. sericata* it is a distinct blue-green. Although the distinction holds in a general fashion, exceptions are far too numerous for it to be at all reliable both for Australian *L. cuprina* and *L. sericata* and for South African specimens of these species seen by the authors.

As a result of examining many hundreds of well-preserved *L. cuprina* and *L. sericata* the following distinguishing features can be listed (Table 1):

Characters on the head

(1) *Hairiness of Central Region of Occiput*.—The occipital region consists of a central area (the cerebrale) and on either side a lateral area separated from the cerebrale by a distinct suture (Figs. 1 and 2). Whereas the lateral areas carry many hairs, the cerebrale is nearly bare, although on its upper part there are some hairs which provide a means of distinguishing *L. cuprina* from *L. sericata*.

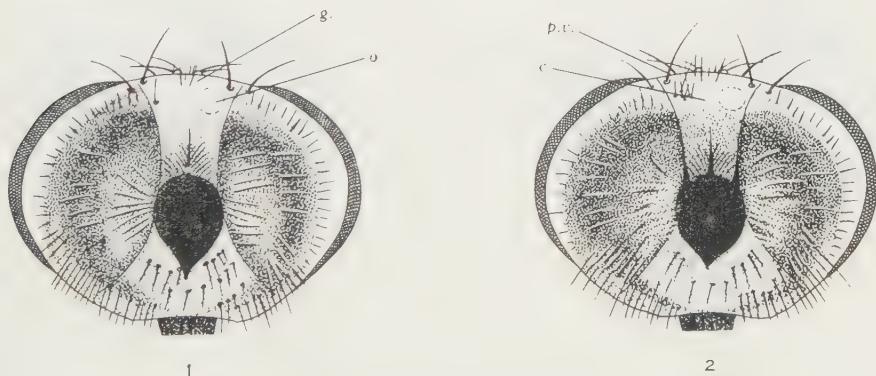


Fig. 1.—Posterior view of head of *L. cuprina*.

Fig. 2.—Posterior view of head of *L. sericata*.

c., cerebrale; g., guide hairs; o., area of cerebrale bearing occipital hairs useful in distinguishing *L. cuprina* and *L. sericata*; p.v., postvertical bristles.

In both *L. cuprina* and *L. sericata* there is a single pair of "guide hairs" on the upper part of the cerebrale directly behind the ocellar triangle^o, the

^o Occasionally one or more additional, but smaller, hairs may be present in this region.

distance between these hairs being nearly equal to that between the posterior ocelli. In *L. cuprina* there is, a little lower down the cerebrale, a second pair of hairs separated by a space several times as great as that between the first pair. At times (less than 20 per cent. of individuals) one or both of the hairs of the second pair is accompanied by a second hair which is, however, often smaller than the first.

In *L. sericata* the second pair of hairs is replaced on each side by a group, each group normally consisting of six to eight hairs, although as few as three and as many as fourteen may be present. The number is, however, frequently different on either side of the same insect. Very occasionally the number of hairs is reduced to two on one or on both sides.

A magnification of 20x to 40x is required for the accurate determination of these occipital hairs. They are seen most readily if the head is viewed from above and to one side and only very rarely and in badly damaged specimens are they broken. This character is extremely reliable.

(2) *Degree of Sclerotization of Clypeus.*—On its dorsal surface the proboscis is flexed between the clypeus and the frons, and the clypeus being bounded laterally by the rostrum (Figs. 3 and 4). It is difficult to see the whole of the clypeus unless the proboscis is extended, which is not often the case in pinned specimens. However, if the insect is turned in such a way that the upper part of the mouth cavity is visible, sufficient of the frontal margin of the clypeus can almost always be seen to enable determination of the character described below.

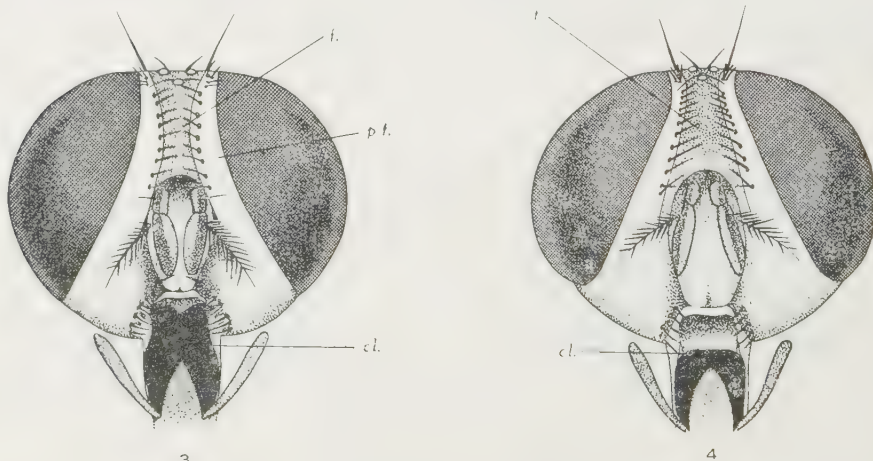


Fig. 3.—Anterior view of head of male *L. cuprina*.

Fig. 4.—Anterior view of head of male *L. sericata*.

cl., clypeus; f., frontal stripe; p.f., parafrontal.

In *L. cuprina* the clypeus, which is slightly bifurcated at its frontal margin, is jet black or a very dark brown, in contrast with the light amber coloration of the rostrum. In *L. sericata* the clypeus is not as distinctly bifurcated anteriorly and is often a light amber colour. However, in a high proportion

of individuals (particularly in males), there are all degrees of sclerotization between amber and dark brown. When the clypeus exhibits its darker shades it is noticeable that its upper half (adjoining the frons) is generally lighter in colour than the lower half.

In spite of the variability of this character it is useful for the rapid initial separation of the two species because it can be employed at low magnifications (10x to 20x). When the sclerotization of the clypeus is such that the specimen cannot be assigned immediately either to *L. cuprina* or *L. sericata*, one or more of the other characters must be used.

(3) *Width of Frontal Stripe*.—The region between the antennae and the ocelli is divided into three areas, namely a median frontal stripe, on either side of which lie the parafrontals (Figs. 3 and 4). The feature distinguishing the two species is the relative widths of the frontal stripe and parafrontals at a level midway between the base of the antennae and the anterior ocellus.

In *L. cuprina* the frontal stripe at this level is equal to, or a little broader than, the width of a parafrontal. In *L. sericata* the frontal stripe is considerably broader (generally at least twice as broad) than either of the parafrontals. Confusion sometimes arises in female *L. cuprina* which occasionally may have the frontal stripe almost twice as wide as a parafrontal. It is, however, a reliable character for males.

Characters on the thorax

(4) *Hairiness of the Lateral Areas of the Mesonotum* (Figs. 5, 6, and 7).—The mesonotum consists of a middle region, on each side of which are two lateral areas. The anterior, somewhat semicircular, areas are sometimes known as the "humeral calli" and the posterior triangular areas as the "notopleura."

Humeral calli.—Arranged transversely across the humeral calli (Figs. 6 and 7) there are three or four large bristles. On the area posterior to these there are, in *L. cuprina*, two or four (rarely up to eight) hairs, whereas in *L. sericata* there are typically six to eight hairs, although all numbers between four and thirteen may be found. Although these hairs can be seen very easily in well-preserved specimens (magnification 30x to 40x), this portion of the body is often rubbed and the bristles and hairs broken off.

Notopleura.—Near the posterior edge of each notopleuron (Figs. 6 and 7) there is a large bristle which can be used to demarcate a narrow region lying between it and the posterior border of this sclerite. In *L. cuprina* there are three to five hairs on this area, whereas in *L. sericata* there are not only eight to sixteen hairs, but these are longer than those of *L. cuprina*. Furthermore, the remaining area of the notopleuron is more sparsely clothed with hairs of shorter length in *L. cuprina* than in *L. sericata*. It is convenient, however, to restrict the area examined to that demarcated above, since this area is less frequently damaged than the remainder of the notopleuron. A magnification of about 40x is required for examination of this character.

(5) *Length of the Second Pair of Presutural Acrostichal Bristles on Mesonotum*.—In *L. cuprina* the second pair of acrostichal bristles (not the dorsocentrals) is much shorter than in *L. sericata*. As a ready means of assessing this, they are, for instance, distinctly shorter than the postvertical bristles (situated lateral to the posterior ocelli (Fig. 2)) and also distinctly shorter than the distance between their point of insertion and the point of insertion of the first pair of postsutural acrostichal bristles (Fig. 5A). In *L. sericata*, the second pair of acrostichal bristles is about the same length as the postvertical bristles and they extend back at least as far as the first postsutural acrostichals (Fig. 5B). This character is very easy to use in well-preserved specimens, but suffers from the disadvantage that the bristles are often broken.

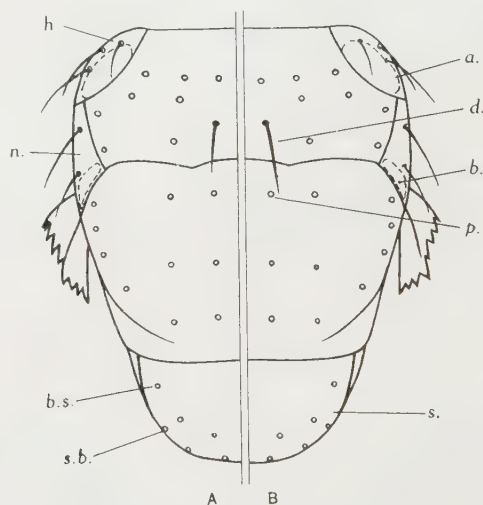


Fig. 5.—Diagram of dorsal aspect of thorax of *L. cuprina* (A) and *L. sericata* (B).

a., area of humeral calli bearing hairs useful in distinguishing *L. cuprina* and *L. sericata*; *b.*, area of notopleuron bearing hairs useful in distinguishing *L. cuprina* and *L. sericata*; *b.s.*, basal bristle; *d.*, second pair of presutural acrostichal bristles; *h.*, humeral calli; *n.*, notopleura; *p.*, first pair of postsutural acrostichal bristles; *s.*, scutellum; *s.b.*, sub-basal bristle.

(6) *Length of Bristles on the Scutellum*.—On the lateral margin of the scutellum there are a series of stout bristles. Between the two anterior bristles (basal and sub-basal) on either side (see Fig. 5) there are a number of hairs. The feature distinguishing the two species is the relative lengths of these hairs and of those on the dorsal surface of the scutellum. In *L. cuprina* the latter hairs are slightly smaller than, or about equal in length to, the lateral hairs. In *L. sericata* the lateral hairs are considerably longer than the dorsal hairs.

(7) *Colour of Fore Femora*.—In *L. cuprina* the exterior surface of the femora of the forelegs is characteristically a very distinct metallic green. This is sometimes modified by a coppery or bronzy sheen and less frequently by a bluish sheen. In *L. sericata* the fore femora are dull, with rarely a dark blue metallic sheen.

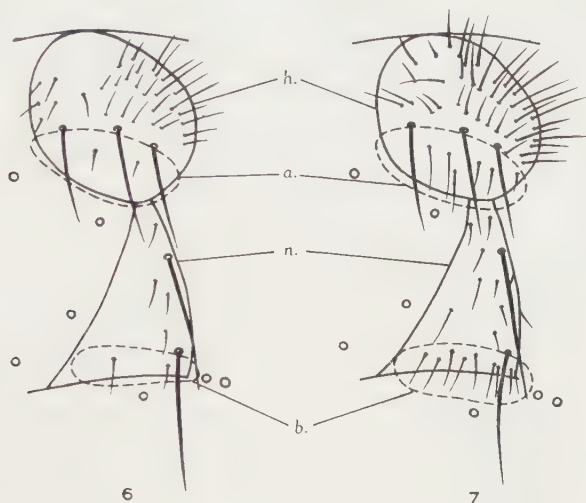


Fig. 6.—Diagram of right lateral area of thorax of *L. cuprina*.

Fig. 7.—Diagram of right lateral area of thorax of *L. sericata*.

a., area of humeral calli bearing hairs useful in distinguishing *L. cuprina* and *L. sericata*; *b.*, area of notopleuron bearing hairs useful in distinguishing *L. cuprina* and *L. sericata*; *h.*, humeral calli; *n.*, notopleura.

This is generally a useful and highly reliable character except when specimens of *L. cuprina* have been killed too soon after emergence (when full coloration has not been attained). It is also difficult to use when specimens have been wet with plant or animal juices, or have been exposed to high temperatures. Under these circumstances the colour of the fore femora is duller and darker than in typical specimens and less easy to distinguish from *L. sericata*.

Characters on the abdomen

(8) *Hairiness of Abdominal Sternites*.—This character is of value in separating the males only of the two species. The region involved in this distinguishing character consists of the second, third, and fourth sternites, together with the adjoining margins of the second, third, and fourth tergites. When viewed laterally it can be seen that this region bears a number of stout bristles.

In *L. cuprina* these bristles are much longer than those on the hind femora and tibiae, whereas in *L. sericata* the bristles are about the same length as those

on the hind legs. This character is noteworthy in that it is the only one observed in which there is greater bristle development in *L. cuprina* than in *L. sericata*, but it is not an easy one to use without experience.

(9) *Contour of the Last Abdominal Tergite*.—In dried specimens of *L. cuprina* the contour of the last abdominal tergite is smooth without depressions, whereas in *L. sericata* this tergite frequently has one or more irregular depressions or dints. This character, which is applicable to dried specimens only, apparently depends on the hardness of the tergite and its resistance to distortion after death of the fly. It is unreliable, therefore, in flies which have been killed too soon after emergence and before hardening has been completed.

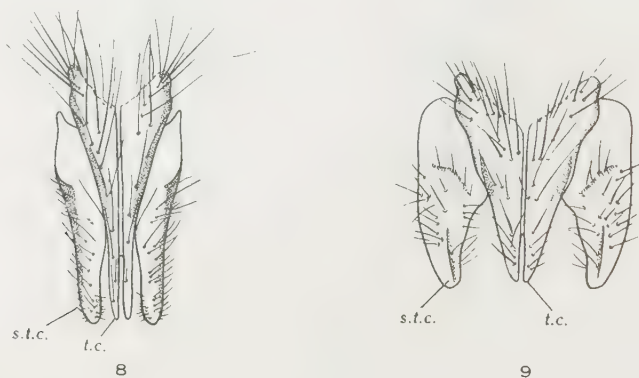


Fig. 8.—Posterior (exterior) view of male genitalia of *L. cuprina*.

Fig. 9.—Posterior (exterior) view of male genitalia of *L. sericata*.

s.t.c., sub-terminal clasper; t.c., terminal clasper.

(10) *Male Genitalia* (Figs. 8 and 9).—Three differences can be seen between the two species in the terminal and subterminal claspers when the dorsal surfaces of these structures are compared:

(i) In *L. cuprina* the terminal claspers are long and slender, the outer margins of the apical third being parallel. In *L. sericata* the terminal claspers are broader and they taper regularly, so that the outer margins of the apical third converge and are not parallel.

(ii) In *L. cuprina* the subterminal claspers are slender and similar in shape to the terminal claspers. In *L. sericata* the subterminal claspers are very much broader than in *L. cuprina*, particularly at their basal half.

(iii) In *L. cuprina* the subterminal claspers are nearly parallel to the terminal claspers, whereas in *L. sericata* the subterminal claspers are nearly semicircular.

(11) *General Appearance*.—In general appearance *L. cuprina dorsalis* is typically a metallic coppery-green, *L. sericata* a bluish green without any coppery sheen. However, there are too many exceptions for this to be a reliable

character. For instance, *L. cuprina* specimens exposed after death to high temperatures (e.g. strong sunlight) often become bluish green and, at times, specimens of *L. sericata*, particularly when freshly killed, have a coppery appearance.

In general, *L. cuprina* is a smaller and more slender fly than *L. sericata*. However, size alone may be deceptive since, due to limitation of larval food, small adults of both species are not uncommon.

Characters of type of L. cuprina cuprina

Except for general coloration (see earlier) Wiedemann's type female of *L. cuprina* agrees with Australian *cuprina* in all the above characters which can be used (K. R. Norris, personal communication). Thus, the hairiness of the central region of the occiput is typical; the clypeus is black; the frontal stripe is about $1\frac{1}{2}$ times the width of a parafrontal; there are two hairs behind the bristles on the humeral calli; the notopleural hairs are typical; and the right fore femur is green. The left fore femur, the presutural acrostichals, and the dorsal hairs on the scutellum are missing.

(ii) *Larvae*

Fuller (1932) has described several features whereby *L. cuprina* and *L. sericata* larvae may be distinguished from one another. Although some of these are useful, others do not appear to be valid. For example, the anterior spiracles of *L. cuprina* were stated to have seven or eight finger-like processes and *L. sericata* ten. In larvae available to us, *L. cuprina* has an average of seven processes (range five to nine) and *L. sericata* has an average of eight (range six to ten). Although this feature can be used to distinguish two populations, it is



Fig. 10.—Lateral view of buccopharyngeal armature of *L. cuprina*.

Fig. 11.—Lateral view of buccopharyngeal armature of *L. sericata*.

m.h., mouth hooks; *v.c.*, ventral cornua.

clearly of limited value in assigning a single larva to a given species. A number of differences were described in the mouth hooks of the two species, but the only constant difference appears to be that the hooks are slightly more curved in *L. cuprina* than in *L. sericata* (Figs. 10 and 11). An apparently constant difference, which was not recorded, but is readily seen, is in the shape of the posterior edges of the ventral cornua. In *L. cuprina* the ventral border is prolonged posteriorly. In *L. sericata* this edge is relatively straight (Figs. 10 and 11). The shape and degree of sclerotization of the posterior spiracles

of the two species (Table 2) is as described by Fuller (1932) but the differences are apparent only to the practised observer. It is clear, however, from Table 2 that there are recognizable differences between the larvae of the two species.

Patton (1920) records six to eight processes in the anterior spiracles of *L. cuprina* from the Orient, whereas Knipling (1936) states that there are four to six in *Phaenicia pallescens* (*L. cuprina*) from North America.

L. sericata is said to have ten to eleven processes in England (Patton and Evans 1929) and five to ten in North America (Knipling 1936).

TABLE 2
SPECIFIC DIFFERENCES BETWEEN AUSTRALIAN *L. CUPRINA* AND *L. SERICATA* LARVAE

Character	<i>L. cuprina</i>	<i>L. sericata</i>
Length (fully grown)	12 mm.	14 mm.
Mouth hooks	Distinctly curved	Rather less curved
Ventral cornua	Posterior edge prolonged ventrally	Posterior edge relatively straight
Posterior spiracles	Roughly oval in shape, peritreme wide and dark	Somewhat pear-shaped, peritreme narrow and not heavily sclerotized
Anterior spiracles	Composed of 7 finger-like processes (av.)	Composed of 8 finger-like processes (av.)

III. GEOGRAPHICAL DISTRIBUTION OF *L. SERICATA* AND *L. CUPRINA*

L. sericata is almost cosmopolitan, having been recorded from all the temperate countries of the world (British Isles, Europe, Africa, Asia, Japan, Australia, New Zealand, North and South America). Hall (1948), however, states that no specimens were collected in Central or South America in 1942 and 1943, nor on any of the islands of the central or south-west Pacific in 1944 and 1945, although over 100,000 flies were collected in these areas. This may be because the regions concerned are unfavourable for *L. sericata* or because this species has not been introduced. It is interesting to note, for example, that Miller (1939) concluded that *L. sericata* has become common in New Zealand only since the closing years of last century. Experience in Australia (see later) suggests that this species occurs principally in the vicinity of homes, which evidently provide suitable requirements often absent elsewhere.

L. cuprina occurs in North and South Africa, Madagascar, Mauritius, India, Assam, Malaya, Indo-China, Laos, China, Java, Soembawa, Timor, Australia, Fiji, Hawaii, North and South America (Brazil). It has not been recorded from the British Isles, Europe, or New Zealand. In 1949 some thousands of blowflies were trapped by B. A. O'Connor in the vicinity of Suva, Fiji, but not a single *Lucilia* was taken. In general, *L. cuprina* appears to be restricted to the warmer sub-equatorial regions, whereas *L. sericata* not only

occurs in these regions, but extends also into the cooler regions of the world. *L. cuprina* appears to tolerate semi-arid conditions better than *L. sericata*, which is typically found in habitats of high humidity.

IV. DISTRIBUTION OF THE TWO SPECIES ACCORDING TO HABITAT

Mackerras and Fuller (1937) record that *L. cuprina* is very widespread in Australia. It occurs in all mainland States (Fig. 12), but has not yet been recorded from Tasmania. It reaches its greatest abundance in the subtropical and semi-arid regions of northern New South Wales and southern Queensland, particularly during spring and autumn. However, it is abundant also on the



Fig. 12.—Map showing known distribution of *L. cuprina*. The rows of dots in eastern Australia trace the path of a collecting trip on which a blowfly trap was exposed at each stop. They indicate a continuous distribution of *L. cuprina* in the area traversed.

cooler southern New South Wales tablelands, and in mountainous as well as low-lying districts. One of the most important factors limiting its distribution appears to be the presence of susceptible sheep on which to breed (Waterhouse 1947). On the other hand, although *L. sericata* has been recorded from all States, including Tasmania (Fig. 13), it is usually an uncommon fly, except in limited areas, and its distribution has no relation to the distribution of sheep. It is known principally from closely settled districts in the cooler parts of Australia, conditions provided by domestic gardens and associated refuse apparently favouring the maintenance of quite dense populations.

However, it has also occasionally been taken many miles from the nearest permanent habitation.

L. sericata occurs commonly in Canberra gardens and *L. cuprina* in the open savannah woodland, characteristic of much sheep-grazing country, which surrounds Canberra. An examination was made, therefore, of the preferences of the two species for these habitats. The method employed was to count the numbers of the two species caught in similarly constructed and baited traps exposed simultaneously in a garden, in open country, and in intermediate situations.



Fig. 13.—Map showing known distribution of *L. sericata*.

In the first experiment the catch from a trap exposed in a well-watered garden with luxuriant green vegetation was compared with catches from traps placed in an unwatered orchard on the outskirts of Canberra, and in three traps placed in open grazing country (Table 3). In the garden, *L. cuprina* formed less than 1 per cent. of the *Lucilia* specimens trapped, in the orchard only 11 per cent., whereas in open country 82-94 per cent. of the *Lucilia* specimens were *L. cuprina*. This suggested a definite preference of *L. cuprina* for open country and of *L. sericata* for more humid, vegetated situation.

To obtain further data, a second experiment was carried out in which the catch from the garden was compared with that from a trap situated 85 yards away in a dry, open, conifer plantation and with other traps extending 760 yards in roughly a straight line into open country (Plate 1). Three traps were also exposed five miles from the city on a sheep-grazing property. It can be seen (Table 4) that, whereas only about 1 per cent. of *Lucilia* specimens

caught in the garden were *L. cuprina*, the proportion rose to about 40 per cent. less than quarter of a mile away in open country and to 97 per cent. five miles from the city on a sheep property. This general trend was followed closely

TABLE 3
RESULTS OF TRAPPING *LUCILIA* IN DIFFERENT HABITATS (23.xi.48 TO 25.xi.48)

Type of Locality		<i>L. cuprina</i> (%)	Number of <i>Lucilia</i>	
			<i>L. cuprina</i>	<i>L. sericata</i>
Humid garden	No sheep in vicinity	0.7	7	1025
Unwatered orchard adjoining a garden	No sheep in vicinity	11	22	173
Open grassland near irrigated plots	Sheep near trap	82	74	16
Lucerne paddock near irrigated plots	Sheep near trap	88	170	24
Open dry grassland	Sheep near trap	94	98	6

during each of the three trapping periods (Table 4). The numbers of *L. cuprina* caught in traps 1 to 6 were not greatly different and the variation in the percentages of this species in the catches was principally due to the fact

TABLE 4
RESULTS OF TRAPPING *LUCILIA* IN DIFFERENT HABITATS

Trap No.	Type of Locality	Distance from Garden (yds.)	Percentage <i>L. cuprina</i> of total <i>Lucilia</i>			Number of <i>Lucilia</i> caught					
			Period 1	Period 2	Period 3	Period 1		Period 2		Period 3	
			14-17.xii.48 21-23.xii.48 25-28.i.49	21-23.xii.48 25-28.i.49	25-28.i.49	<i>L. cuprina</i>	<i>L. sericata</i>	<i>L. cuprina</i>	<i>L. sericata</i>	<i>L. cuprina</i>	<i>L. sericata</i>
1	Humid garden	0	1.5	1.2	1.6	48	3246	23	1912	21	1256
2	Unwatered plantation	85	8	5	17	35	390	26	300	19	94
3	Grassland within plantation	150	34	27	42	40	79	11	29	7	10
4	Open grassland	390		37	55	—	—	10	17	6	5
5	Open grassland	610	42	42	40	32	45	21	30	4	6
6	Open grassland	760		44	47	—	—	17	22	7	8
7					96					24	1
8	Sheep-grazing property	5 miles			98					42	1
9					97					34	1

that far larger numbers of *L. sericata* were caught in the garden and the plantation (traps 1 and 2) than elsewhere. It appears, therefore, that *L. sericata* has a very definite preference for the conditions prevailing in the garden. *L.*

cuprina, on the other hand, does not exhibit such a clear preference for any of the situations examined.

This is in accord with the work of Gilmour, Waterhouse, and McIntyre (1946) who found that, in the Canberra district at least, the *L. cuprina* population ranged widely and at random over the countryside, individual flies being capable of covering up to about five miles in less than 30 hours. The larger numbers of *L. cuprina* trapped five miles from Canberra than elsewhere (Table 4) are probably due to the presence of sheep in the immediate vicinity of these traps, whereas no sheep were near traps 1 to 6 during, or for some time before, the experimental period. A similar explanation probably holds for the comparatively high catches of *L. cuprina* in the three traps of the first experiment (Table 3) which were exposed in the vicinity of sheep. Because sheep are specifically attractive to *L. cuprina* and particularly to the gravid female of this species (Mackerras and Mackerras 1944), one would expect a somewhat higher density of these flies near sheep than elsewhere. Furthermore, any struck sheep would result, at least temporarily, in a considerable local increase in the numbers of *L. cuprina*.

Cragg (unpublished data) found that, in North Wales, *L. caesar* is present in highest density along hedgerows and that the rate of dispersal of a population is comparatively slow. It may be inferred from the present experiments that in Australia *L. sericata* behaves similarly. In Canada and U.S.A., *L. sericata* frequents urban districts, where it is common on almost any kind of garbage, especially when this contains meats or damaged fruit. It frequently rests on leaves of vegetables and shrubs and is attracted to honeydew (Hall 1948). Its observed behaviour in Australia is similar to that recorded from America.

V. HYBRIDIZATION EXPERIMENTS

Successful crosses between *L. cuprina* and *L. sericata* were made by Mackerras (1933), but no details are given except that a F_1 male and a F_1 female from a male *L. cuprina* $\times$ female *L. sericata* mating were both indistinguishable from typical *L. cuprina*. These F_1 hybrids were used in a fertility experiment, one particular female laying 3171 eggs. Since this was considerably more than usually obtained either from pure *L. cuprina* or pure *L. sericata* it was suggested that hybrid vigour might be responsible. The results were not published in any further detail because, by the criteria then available (coloration of fore femora and structure of male genitalia) all flies of F_1 and F_2 generations of this and the reciprocal cross exhibited only *L. cuprina* characters, a result which could not be explained on the basis of any known behaviour of genes. The only other information available on these experiments is that the original species crosses produced few eggs, which seemed to support the idea that this was an unusual cross (M. J. Mackerras, personal communication).

Some of the flies from these crosses are still available for study. These were examined using the characters described earlier. All the flies (Table 5) of the F_1 and F_2 generations are typical *L. cuprina*.

In an endeavour to check these extraordinary results, pupae from pure *L. cuprina* and *L. sericata* cultures were separated into individual tubes a day or two before emergence and the adults subsequently sexed and set up in cages

TABLE 5

FLIES AVAILABLE FOR EXAMINATION FROM MACKERRAS'S (1933) *L. CUPRINA* × *L. SERICATA* CROSSES

Parents		F ₁		F ₂	
Male	Female	Male	Female	Male	Female
<i>L. cuprina</i>	<i>L. sericata</i>	1	1	124	142
<i>L. sericata</i>	<i>L. cuprina</i>	1	1	5	5
<i>L. sericata</i>	<i>L. cuprina</i>	1	1	—	—
<i>L. sericata</i>	<i>L. cuprina</i>	—	11	—	—
<i>L. sericata</i>	<i>L. cuprina</i>	9	1	71	26

as shown in Table 6. Fresh liver was provided daily and each cage was supplied continuously with sugar and water. The cultures were kept at about 25°C. and 30 per cent. relative humidity, conditions under which both species breed readily.

TABLE 6

DETAILS OF *L. CUPRINA* AND *L. SERICATA* CROSSES

Parents		Type of Experiment	No. of Tests	No. of <i>L. cuprina</i> per Experiment	No. of <i>L. sericata</i> per Experiment	Result
Male	Female					
<i>L. cuprina</i>	<i>L. sericata</i>	Single pairs	29	1	1	No larvae
		Communal cages	1	6	6	No larvae
		Communal cages	1	35	3	No larvae
		Communal cages	1	5	1	No larvae
		Communal cages	1	10	9	No larvae
		Communal cages	1	14	10	No larvae
		Communal cages	1	21	32	No larvae
		Communal cages	1	7	14	No larvae
<i>L. sericata</i>	<i>L. cuprina</i>	Single pairs	8	1	1	No larvae
		Communal cages	1	9	8	No larvae
		Communal cages	1	9	9	No larvae
		Communal cages	1	16	16	
		Communal cages	1	50	25	290 F ₁ adults

Eggs were laid in a number of these negative experiments, but failed to hatch. The amount of oviposition was no greater, however, than would have been expected from unfertilized females.

In the only successful cross 25 male *L. sericata* and 50 female *L. cuprina* were kept in a 12 × 12 × 12 in. cage. Although 10 egg masses were laid

over a period of about a month, larvae were produced on only seven occasions, resulting in 151 male and 139 female F_1 hybrids. Since the number of offspring per female from an intra-species cross generally lies between 300 and 500 the small number of progeny (290) obtained indicates that the cross was not a very successful one. Perhaps only one of the 50 females was responsible for laying the fertile eggs.

The reciprocal cross (21 male *L. cuprina* $\times$ 32 female *L. sericata*) carried out at the same time under identical conditions produced eggs on many occasions, but none hatched.

The 75 parents and 290 F_1 hybrids from the successful cross were examined individually for all characters except male genitalia. All the parents were typical of the particular species to which they belonged (Table 6). The results of the examination of hybrids may be summarized as follows:

Characters in which *L. cuprina* was usually dominant:

- (a) Sclerotization of clypeus (generally black or dark brown).
- (b) Coloration of fore femora (about 5 per cent. of individuals had legs which were duller than typical *L. cuprina*).
- (c) Length of ventral abdominal bristles in the male.
- (d) Length of bristles on the scutellum.

Characters in which *L. sericata* was sometimes dominant:

- (a) Length of second pair of presutural acrostichal bristles (often nearly as long as in *L. sericata*, but many intermediates occurred).
- (b) Width of frontal stripe in male.
- (c) Width of frontal stripe in female (although there were many intermediates).
- (d) Contour of the last abdominal tergite.

Characters generally intermediate between the two species (see Table 7):

- (a) Occipital bristles (about 10 per cent. indistinguishable from *L. cuprina*, but less than 1 per cent. indistinguishable from *L. sericata*).
- (b) Hairiness of humeral calli.
- (c) Hairiness of notopleura.

About 80 per cent. of the F_1 hybrids had characters or combinations of characters which clearly placed them as intermediates between *L. cuprina* and *L. sericata* but generally closer to *L. cuprina*. About 10 per cent. were indistinguishable from *L. cuprina* and the remaining 10 per cent. distinguishable only with varying degrees of difficulty. No F_1 hybrids were found that could be confused with *L. sericata*. Although the "dominant" characters of *L. cuprina* and *L. sericata* generally appeared together in the progeny, there did not appear to be any close linkage between any of the characters examined, since any character at times varied independently of any other.

F_2 progeny were obtained from three of the seven batches of F_1 hybrids. One hundred and forty-eight F_2 males and 117 F_2 females from three separate ovipositions of one of these three F_1 batches were examined and all were found

to be indistinguishable from typical *L. cuprina*. On the other hand, of 25 F_2 males and 31 F_2 females from the other two batches of F_1 hybrids that laid eggs, one male and six females were indistinguishable from *L. cuprina*, three males and one female were indistinguishable from typical *L. sericata*, and the remainder were intermediates, although many of the latter only differed from one or other species by a single character.

TABLE 7
AVERAGE NUMBER OF HAIRS (AND UPPER AND LOWER LIMITS) ON THREE REGIONS OF
L. CUPRINA, *L. SERICATA*, AND F_1 HYBRIDS

Species	Sex	Occipital Hairs	Humeral Calli	Notopleura
<i>L. cuprina</i>	Male	1 (0-2) (5% with 2 on one side)	2 (0-8)	3 (2-5)
<i>L. cuprina</i> (parents)	Female	1 (1-3) (20% with 2 on at least one side); (1 with 3 on one side)	2 (0-7)	3 (1-5)
<i>L. sericata</i> (parents)	Male	6 (4-9)	8 (6-13)	11 (9-16)
<i>L. sericata</i>	Female	8 (5-14)	6 (4-12)	11 (9-15)
F_1	Male	2 (1-5)	5 (1-11)	7 (2-11)
F_1	Female	2 (1-6)	3 (1-7)	5 (4-9)

Summing up, it is clear that there is considerable difficulty in obtaining successful matings between *L. cuprina* and *L. sericata*. Most of the F_1 hybrids exhibited characters of both species, although some could not be distinguished from *L. cuprina*. The picture in the F_2 generation is very complicated. Some entire F_2 batches are typical of *L. cuprina*, whereas others are mainly intermediates. Further work is required before an explanation can be advanced for this result and for the results of Mackerras.

General confirmation of our findings comes from some material* of Ullyett's crossing experiments, which have been outlined earlier (Ullyett 1945). The specimens comprised 12 male *L. cuprina*, 9 female *L. sericata*, which were used as parents, and 7 male and 6 female F_1 hybrids. The male *L. cuprina* were typical, except that the frontal stripe was rather more divergent ventrally than usual (i.e. they tended slightly towards *L. sericata* in this respect). The abdominal coloration was indistinguishable from that of Australian male *L. cuprina* and *L. sericata* and varied from coppery to green. Six of the female *L. sericata* were typical; the wings of the remaining three were crumpled and the ptilinum had not been properly retracted. There was no blue coloration whatever of the abdomen. The hybrids were typical of *L. cuprina* in all our characters except the degree of sclerotization of the clypeus, which was typical of *L.*

* Made available by courtesy of the Division of Entomology, Pretoria.

sericata. All must therefore be regarded as intermediates. The abdominal coloration of these hybrids was a deeper bluish green than that of their parents and the first visible segment had a bluish sheen in most specimens, but not all. This may be due to different conditions of drying (they were pinned 11 days after their parents), since the authors have found that specimens tend to become bluish if they are dried at higher temperatures than usual. No support whatever could be found from these specimens for Ullyett's contention that abdominal coloration is useful for distinguishing between *L. cuprina* and *L. sericata*.

VI. RELATION OF THE TWO SPECIES TO BLOWFLY STRIKE

(a) *Attractiveness of Sheep to the Two Species*

There is good evidence from insectary experiments that *L. sericata* does not lay its eggs on sheep as readily as does *L. cuprina* (Mackerras and Mackerras 1944). In one experiment eight sheep were exposed for a week in an insectary to gravid *L. sericata*, eggs being laid on two of the sheep. Some *L. cuprina* were then added to the insectary, and, within four hours, all eight sheep had eggs laid on them, indicating that the sheep were more attractive to *L. cuprina* than to *L. sericata*. Some of the freshly laid eggs were collected from each sheep and allowed to develop. From seven of the eight sheep these eggs produced both *L. cuprina* and *L. sericata* and from the eighth sheep only *L. cuprina*. It is clear, therefore, that oviposition by *L. cuprina* stimulated *L. sericata* to lay eggs on sheep which they had previously ignored.

In another experiment three sheep were exposed in turn in a small cubicle to a high density of mature *L. sericata*. After about five hours, when no eggs had been laid, each was transferred to a similar density of *L. cuprina* in another cubicle, eggs being laid on each sheep within two hours. When similar sheep were exposed first to *L. cuprina*, oviposition followed in the first few hours (Mackerras and Mackerras 1944).

Cragg (1950a) has also obtained results with *L. sericata* in Great Britain under field conditions which indicate that this species and Australian *L. cuprina* show marked differences in behaviour.

(b) *Importance of the Two Species in Causing Strike*

Records of the occurrence of the two species in field strikes in Australia (Table 8) show quite clearly that *L. cuprina* is far more important than *L. sericata* in producing strike wounds. In the Australian Capital Territory, from which the most strikes were examined, 58.3 per cent. of the *L. cuprina* strikes contained no other species, while only 3.8 per cent. of the *L. sericata* strikes contained this species alone. *L. sericata* was present in significant numbers in one year only. The relatively small number of *L. sericata* strikes and the small percentage in which this species alone occurs is doubtless due to two factors. One is the comparatively low population density of this species in open country and the second is the fact that it is not as powerfully attracted as *L. cuprina*

to lay its eggs on sheep. Its presence in strikes principally from around Canberra may be a result of the fact that sheep are grazed closer to gardens here than is common in other parts of Australia.

L. cuprina is the principal cause of blowfly strike in South Africa (Hepburn 1943; Monnig and Cilliers 1944) and it has also been recorded as attacking sheep in Kenya (Lewis 1933). It is apparently of no economic importance in North America as far as sheep are concerned (Hall 1948), nor is it a serious sheep pest in the Orient, possibly owing to the presence of relatively few susceptible sheep.

TABLE 8
DETAILS OF STRIKES IN WHICH *LUCILIA* LARVAE WERE PRESENT
(FROM MACKERRAS AND FULLER 1937; MACKERRAS AND MACKERRAS 1944)

State	Number of Strikes	
	<i>L. cuprina</i>	<i>L. sericata</i>
Queensland	197	0
New South Wales	242	5
Australian Capital Territory	696	79
South Australia	196	0
Western Australia	175	0
Total	1506	84

L. sericata is the principal sheep blowfly of the British Isles (Davies 1934; Macleod 1943; Ratcliffe 1934) and is one of the two important sheep blowflies of New Zealand (Miller 1939). Blowfly strike of sheep by *L. sericata* occurs also in South Africa (Hepburn 1943) and in U.S.A. (Bishopp 1915), but this species is not a serious pest in either country. *L. sericata* does not appear to attack sheep at all commonly in eastern Europe (Cragg 1950*b*) or in the Ukraine or in Northern Caucasus (Paramonov 1937), although it is not an uncommon fly in these regions.

VII. DISCUSSION

It is abundantly clear from the evidence presented that the flies known in Australia as *L. cuprina* and *L. sericata* are distinct species. Not only are there many constant morphological differences between the adults, but the larvae can also be distinguished; hybridization experiments suggest that there is often considerable difficulty in obtaining successful matings; examination of several thousand wild *Lucilia* failed to reveal any intermediates; ecological studies show that the two species have different habitat preferences and that their geographical distribution is not alike; their degree of attraction to living sheep is very different; and finally there are physiological differences.

One result of these physiological differences is the larger size of both larvae and adults of *L. sericata* when this species and *L. cuprina* are bred in an identical fashion and provided with adequate food. Further, the duration of the developmental period from egg to adult is significantly longer in *L. sericata* (14-15 days at 25°C.) than in *L. cuprina* (12-13 days).

Many interesting problems have arisen in the course of this survey of the two species. What, for example, are the factors which restrict the spread of *L. sericata* from the vicinity of dwellings into the surrounding country, and where do they breed? When one or two hundred *L. sericata* were trapped on each of several consecutive days in the Canberra garden (Tables 2 and 3) there was no apparent fall in the numbers of wild *L. sericata* remaining. In this area there could scarcely have been sufficient dead vertebrates (none were known to exist at all) to maintain a relatively steady population, apparently equivalent to between 500 and 1000 *L. sericata* per acre. It would be interesting to determine whether this species can maintain a high population density by breeding in vertebrate excrement, particularly since it has been recorded in small numbers from pig manure (Thomsen 1938) and both it and *L. cuprina* from fowl manure (Illingworth 1923; Tanada, Holdaway, and Quisenberry 1950). Other possible breeding grounds are small invertebrates (e.g. dead snails or fleshy insects) and vegetable material. *L. cuprina* is thought not to be able to maintain a high population density in the absence of sheep (Waterhouse 1947), and even in typical sheep country near Canberra its population density is comparatively low, densities of 0.3 to 5.7 per acre being recorded at different times of the active sheep blowfly season (Gilmour, Waterhouse, and McIntyre 1946).

Another question which arises is whether or not *L. cuprina cuprina* and *L. cuprina dorsalis* are really quite distinct in spite of the fact that there do not appear to be any constant morphological differences which would enable a taxonomist to assign specific status to them. The most striking differences are in general coloration and in the fact that, in North and South America, which are the only important sheep-raising countries falling within its distribution, *L. cuprina cuprina* is not an important sheep pest. The latter might be due to the fact that American sheep are relatively insusceptible to blowfly strike, although this is improbable in view of the common occurrence in some regions of the wrinkly Vermont breed.

From South Africa the bulk of the evidence on habits and behaviour of *L. cuprina* and *L. sericata* and also a morphological comparison of specimens from that country and from Australia suggest that the respective *Lucilia* species are very similar, perhaps even identical, in the two countries. There is as yet insufficient evidence to decide whether or not *L. sericata* from England and Australia are similar in physiology and behaviour as well as in morphology.

Finally, the hybridization experiments indicate a most unusual state of affairs, which does not appear to be at all clear on the basis of simple Mendelian laws. Although the early results of Mackerras could not be substantiated in detail because of difficulties of securing successful crosses, our one fertile mating demonstrated that it was possible to obtain at least some F_1 hybrids which were apparently typical *L. cuprina* and also that the entire F_2 progeny from some (but not all) F_1 hybrids appeared to be typical *L. cuprina*. Mackerras (1933) found that *L. cuprina* characters were dominant even if the original male parent was *L. cuprina* and the female *L. sericata*, a cross which

the authors could not repeat. A comparable result does not appear to have been recorded before, although it is known from mosquitoes that the characters of the female may appear in all offspring. For example, when *Aedes albopictus* males are mated with *A. aegypti* females, the F_1 and F_2 progeny have *A. aegypti* characters (7 features examined) (Downs and Baker 1949; Toumanoff 1937), but if male *A. aegypti* are crossed with female *A. albopictus* the F_1 and F_2 progeny are typical of *A. albopictus* (Hoang-Tich-Try 1939; Toumanoff 1937, 1939). Crosses between *L. cuprina* and *L. sericata* appear to be well worthy of further attention by the geneticist.

It has been suggested that *L. cuprina* was introduced into Australia, and the history of the spread of blowfly strike supports this view (Joint Blowfly Committee 1933). More recent evidence lends further support. For instance, the gradual spread of this species up the coast of Western Australia, which was recorded by Mackerras (1936) and Mackerras and Fuller (1937) is apparently still continuing (Jenkins 1945). Thus strike, believed to be due to *L. cuprina*, was first recorded at Mt. Anderson (near Derby) in 1942, although at that time properties further east in the Fitzroy basin had experienced no trouble. Furthermore, there are other corroborative reports, such as from Nappamerrie station (extreme south-western Queensland), where sheep were run from 1900 to the late 'twenties with no blowfly trouble. In the late 'twenties, successive severe waves of strike caused sheep-raising to be abandoned in this district. However, as recently as November 1949, *L. cuprina* was taken at Nappamerrie, so that it is evidently able to maintain a population for 20 years or more in the absence of sheep. It is most unlikely that there would have been some 30 successive strike-free years if *L. cuprina* had always occurred in this region, as would be expected if it were a native species.

There is now no support for the view (Joint Blowfly Committee 1933) that *L. cuprina dorsalis* was introduced into Australia from the East (i.e. Eastern Asia, or the islands to the north of Australia), since it is not known to occur in this region. It now appears more probable that it was introduced from South Africa or India during the last century. The fact that there are no records of strike in Australia before 1883 (Joint Blowfly Committee 1933) does not necessarily mean that *L. cuprina dorsalis* was introduced about that time, since the first strikes were recorded in South Africa about 1900 (Smit 1931), although *dorsalis* (Robineau-Desvoidy 1830) and *argyrocephala* (Macquart 1846) had been present for at least 70 years before that time. If *L. cuprina cuprina* is found to occur in Northern Australia (and this possibility cannot be ruled out without further collections from this region) it would be circumstantial evidence in support of the view that *L. cuprina dorsalis* is an introduced species.

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Aerial photograph showing the distribution of traps (indicated by white triangles) in the habitat preference experiment.

THE CATALYTIC EFFECT OF MOLYBDATE ON THE BREAKDOWN OF PHOSPHOCREATINE*

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Summary

The hydrolysis of phosphocreatine in 0.1N HCl at 37 and 65°C. yields, as the principal products, creatine and orthophosphoric acid. Under the influence of molybdate, phosphocreatine in 0.1N HCl at 37 and 65°C. breaks down into creatinine, creatine, and orthophosphoric acid. The relative proportions of the two former compounds are a function of the molybdate concentration. Increase of pH leads to a progressive reduction in this effect of molybdate, which apparently depends upon the degree of ionization.

Of other compounds investigated, only sodium vanadate had a similar effect and then only in a concentration ten times greater than that of ammonium molybdate.

I. INTRODUCTION

The catalytic effect of molybdate on the acid hydrolysis of phosphocreatine was first described by Fiske and Subbarow (1929) but the products of this hydrolysis were not identified. It is generally supposed that these would be creatine and orthophosphoric acid although Meyerhof and Lohmann (1928) were unable to detect the former by the diacetyl method of Walpole (1911). It was concluded that the creatine liberated was so changed in the presence of molybdate that it no longer gave the diacetyl reaction. The most obvious change which creatine could undergo under such conditions is conversion to creatinine, but in view of the tendency of phosphocreatine to ring closure the formation of creatinine without the intermediate formation of creatine must be borne in mind. The possible reactions involving the hydrolysis of phosphocreatine in acid solution and in the presence of molybdate may be represented as in Figure 1.

The possibility of reaction (I) occurring in the presence of molybdate is not ruled out by the results of Meyerhof and Lohmann (1928). The formation of creatinine from creatine—reaction (II)—occurs in acid solution. Reaction (III), involving the loss of phosphoric acid across the carboxyl group with simultaneous ring closure, apparently occurs in the solid state. Thus Zeile and Meyer (1938) have shown that the calcium salt of phosphocreatine when heated in vacuo and in the presence of dry HCl is transformed into creatinine.

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Such a reaction also occurs in aqueous solution and has formed the basis of the hypothesis developed by Borsook and Dubnoff (1947) to account for the formation *in vivo* of creatinine from phosphocreatine by a non-enzymic reaction. The amounts of creatinine which were formed under the conditions used by these workers were small and would not account for the negative

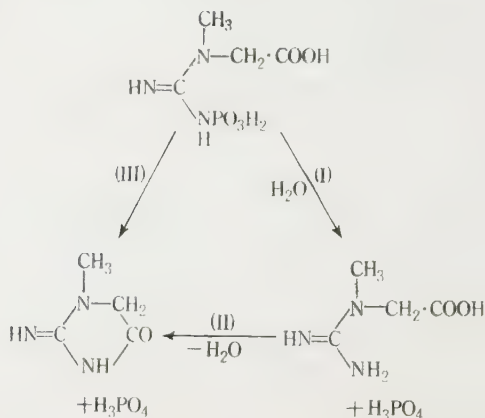


Fig. 1.—Reactions involving hydrolysis of phosphocreatine.

creatine tests reported by Meyerhof and Lohmann (1928). Lundquist (1947) has reported that under more severe conditions (in the presence of HCl or "8/9 saturated picric acid") a small proportion of phosphocreatine is transformed directly into creatinine. No reference has been found to the effect of molybdate on this reaction. It is the purpose of this communication to report the results of investigations designed to show that, under the influence of molybdate, phosphocreatine is transformed into creatinine without the intermediate formation of creatine.

II. MATERIALS AND METHODS

Phosphocreatine.—A preparation of the crystalline sodium salt synthesized as described by Ennor and Stocken (1948). All solutions were prepared immediately before use and the pH adjusted to approx. 7.3 by the addition of 0.1N NaOH.

Ammonium molybdate.—Reagent grade.

Inorganic phosphate.—A modification by Ennor and Stocken (1950) of the Berenblum and Chain (1938) method was used. This modification makes possible the accurate and direct determination of inorganic phosphate in the presence of phosphocreatine.

Creatine.—The method described by Eggleton, Elsdon, and Gough (1943) was used with minor modifications.

Creatinine.—Determined by the alkaline picrate method.

III. RESULTS

As it has been pointed out by Lundquist (1947) that, during the acid hydrolysis of phosphocreatine, there is some formation of creatinine, the initial experiments were designed to determine the mildest conditions which would effect complete hydrolysis and produce minimal amounts of creatinine. Experimentally it was found that these conditions were exposure to 65°C. for 9 minutes in the presence of 0.1N HCl (Table 1).

It was not possible to reduce the amount of creatinine formed and yet attain complete hydrolysis by variation of the time of hydrolysis, pH, and temperature. The effect of molybdate on such a hydrolysis was then determined in a parallel experiment. The technique adopted was to add, at zero time, 2.0 ml. of an approximately $1.5 \times 10^{-3}M$ solution of sodium phosphocreatine to each of two tubes (one containing 2.0 ml. of 0.4N HCl and 4.0 ml. of water, and the other 2.0 ml. of 0.4N HCl, 1.0 ml. of $1 \times 10^{-3}M$ ammonium molybdate, and 3.0 ml. water) both of which had been equilibrated at 65°C. At zero + 9 min., 2.0 ml. of 0.4N NaOH was added to each tube, the contents mixed, and cooled in an ice bath. Suitable aliquots were then withdrawn for the determination of P, creatine, and creatinine. As a check on the possible effect of molybdate on creatine in acid solution, 2.0 ml. of a solution containing 35 $\mu g.$ of creatine and 30 $\mu g.$ of P/ml. were added to a third tube containing 2.0 ml. 0.4N HCl, 1.0 ml. of $1 \times 10^{-3}M$ ammonium molybdate, and 3.0 ml. of water. This was treated in a similar manner to the other tubes. The results (Table 1) show that, in the absence of molybdate, 10.8 per cent. of the phosphocreatine (expressed as creatine) is converted to creatinine, whereas in the

TABLE 1

PRODUCTS OF HYDROLYSIS OF PHOSPHOCREATINE IN 0.1N HCl AT 65°C. FOR 9 MINUTES IN PRESENCE AND ABSENCE OF AMMONIUM MOLYBDATE (FINAL CONC. = $1.25 \times 10^{-4}M$)

All results are the means of duplicate determinations

	Tube 1 Phosphocreatine No Molybdate	Tube 2 Phosphocreatine Plus Molybdate
$\mu g.$ Creatine	129	60.5
$\mu g.$ P	35.4	35.0
$\mu g.$ Creatinine	13.5	71
$\mu g.$ Creatine $\equiv$ creatinine	15.7	82.2
$\therefore \mu g.$ Total creatine	144.7	142.7
Molar ratio P/creatine	1.03	1.03

In tube 3, to which 70 $\mu g.$ of creatine and 60 $\mu g.$ of P had been added, 70 $\mu g.$ were recovered on analysis and no creatinine detected.

presence of a final concentration of $1.25 \times 10^{-4}M$ molybdate a 57.6 per cent. conversion has occurred. As creatine is unchanged under these conditions, it is evident that, under the influence of molybdate, phosphocreatine is converted directly into creatinine.

The influence of varying concentrations of molybdate on the hydrolysis of phosphocreatine in 0.1N HCl at 65°C. for 9 min. has been determined over the range of 0.025 to 0.5 μ -moles of ammonium molybdate/ml. These results (Fig. 2) indicate the marked influence of molybdate on the amount of creatine

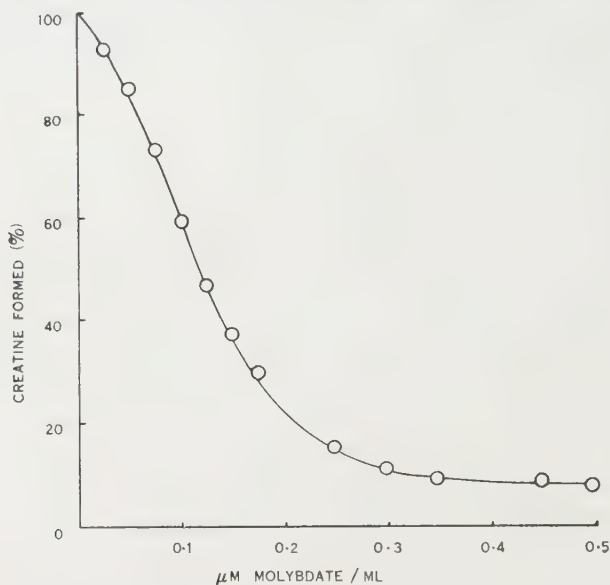


Fig. 2.—Influence of increasing concentration of molybdate ion on the amount of creatine released from phosphocreatine in 0.1N HCl at 65°C. in 9 min. These conditions produce complete hydrolysis. All points on the curve represent the means of at least two determinations. Phosphocreatine conc. 0.13 μ -moles/ml.

released from phosphocreatine under conditions which, even in the absence of this ion, effect complete hydrolysis. There is, however, a pronounced flattening of the curve at the higher concentrations. Thus with 0.4 μ -moles of molybdate/ml., 11.4 per cent. of the possible amount of creatine is formed and this is reduced to only 8 per cent. when the amount of molybdate is increased to 0.5 μ -moles/ml.

It was considered of interest to determine the effect of the molybdate ion upon the hydrolysis of phosphocreatine in 0.1N HCl and under conditions of temperature and time which, in the absence of molybdate did not effect complete hydrolysis. A temperature of 37°C. was chosen for these experiments, and the degree of hydrolysis at the end of each time interval was measured by determining the inorganic P released. The results (Fig. 3) indicate that under these conditions the compound is 50 per cent. hydrolysed in 15 minutes and complete hydrolysis is reached in 90 minutes.

In the experiments employing molybdate a constant hydrolysis time of 30 minutes was chosen as this, in the absence of molybdate, produces incomplete hydrolysis (Fig. 3). The catalytic effect of molybdate upon this hydrolysis is

apparent from Figure 4. In these experiments an experimental technique similar to that employed in the experiments at 65°C. was followed, and the degree of hydrolysis was determined as before.

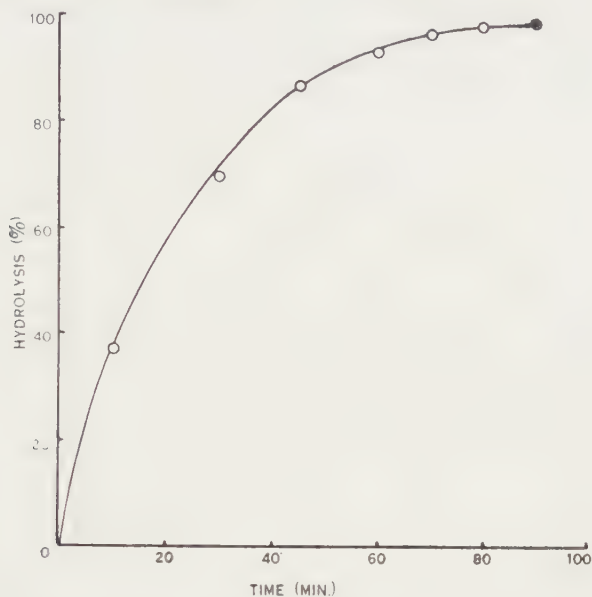


Fig. 3.—Hydrolysis curve of phosphocreatine in 0.1N HCl at 37°C. All points on the curve represent the means of two determinations.

Under these conditions the curve (Fig. 5) illustrating the amounts of creatine produced with varying amounts of molybdate is very similar to that obtained at 65°C. (Fig. 2). It is also of interest to note that, at 37°C. and in the presence of 0.5 μ -moles of molybdate/ml., virtually 100 per cent. hydrolysis occurs in 30 minutes (Fig. 4), and that again conversion to creatinine is not quite quantitative—about 8 per cent. of the possible amount of creatine still appears (Fig. 5). It has been found impossible to obtain quantitative conversion even by increasing the molybdate concentration to 50 μ -moles/ml.

The effect of molybdate upon the course of the hydrolysis of phosphocreatine at varying pH has also been determined. In these experiments a potassium hydrogen phthalate buffer was used at a final concentration of 0.1M. pH values were checked with the glass electrode. The experiments were carried out at 37°C., and the time of hydrolysis was in all cases 30 minutes. As before, the degree of hydrolysis was determined by measurement of the inorganic phosphate released, and, in addition, simultaneous determinations were made of the creatine and creatinine formed. As a check on the analytical methods, the molar ratio—P/total creatine, i.e. creatine + creatine equivalent of creatinine—has been calculated and is included in Table 2. It is clear that, irrespective of the degree of hydrolysis and whether or not molybdate is present, the analytical methods used are capable of illustrating the course of the

hydrolysis with reasonable accuracy. With decreasing pH the percentage of phosphocreatine hydrolysed decreases both in the presence and absence of molybdate. At any one pH the catalytic effect of molybdate is apparent although this effect, like that of increased creatinine formation, disappears above pH 4.0; thus at pH 4.6 and 5.4 there is no greater formation of creatinine when molybdate is present, and the degree to which the phosphocreatine is hydrolysed remains affected only by the pH.

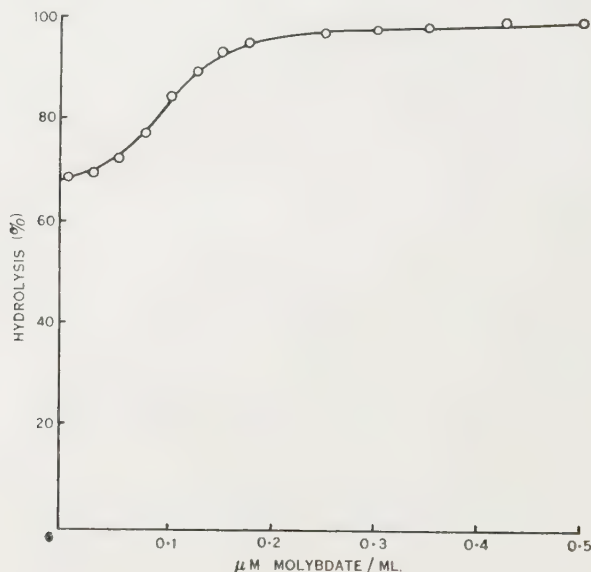


Fig. 4.—Hydrolysis curve of phosphocreatine in 0.1N HCl at 37°C. for 30 min. in the presence of varying amounts of ammonium molybdate. Phosphocreatine conc. 0.13 μ -moles/ml. All points on the curve represent the means of duplicate determinations.

IV. DISCUSSION

The experimental results do not permit of an explanation either of the effect of molybdate as a catalyst in the breakdown of phosphocreatine or of its effect in altering the normal direction of hydrolysis, with the production of larger amounts of creatinine. It seems possible, however, that the reaction depends upon the formation of a molybdate-phosphocreatine complex, as indeed is suggested by the results in Table 2. In this connection it is of interest to note that the hydrolysis of phosphoarginine is actually retarded in the presence of molybdate (Lohmann 1928).

Irrespective of the mechanism involved it is now clear that the failure of Meyerhof and Lohmann (1928) to detect creatine after an acid-molybdate hydrolysis of phosphocreatine was due not to any change in creatine, as was suggested, but to the fact that creatinine, and not creatine, was the principal product of the hydrolysis.

Some experiments have been carried out to determine the effect of other inorganic compounds on the hydrolysis of phosphocreatine. An effect similar to that of ammonium molybdate was found only with sodium vanadate. On an equivalent concentration basis this compound was only about one-tenth as effective as ammonium molybdate.

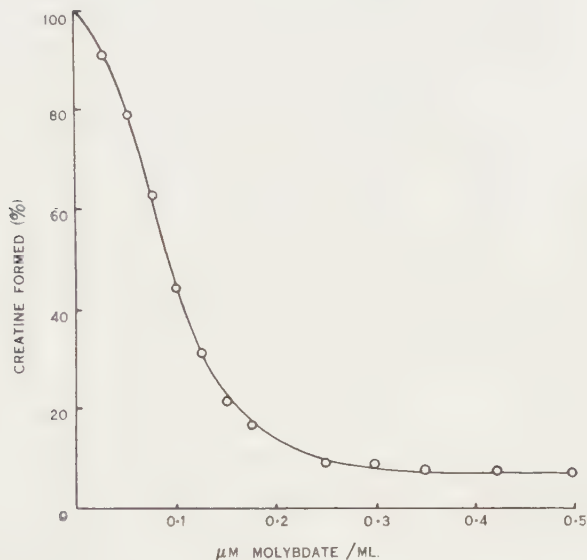


Fig. 5.—Influence of increasing concentration of molybdate ion on the amount of creatine released from phosphocreatine in 0.1N HCl at 37°C. in 30 min. The ordinate gives the amount of creatine released expressed as the percentage of the maximum possible amount under similar conditions of time, temperature, and acid concentration but in the absence of molybdate. All points on the curve represent the means of duplicate determinations.

It seemed possible that the failure of many workers in the past to prepare an enzyme system capable of transforming phosphocreatine to creatinine may have been due to the absence of a coenzyme. Since Mo is a trace metal to which no function in the body has, as yet, been ascribed and because of its effect in a model system *in vitro*, experiments were designed to test the hypothesis that Mo may have such a coenzyme action. These experiments were carried out with dialysed homogenates of skeletal muscle, liver, and kidney, and yielded negative results.

It is possible, since molybdate induces ring closure very readily under extremely mild conditions with phosphocreatine, that it may also be useful in effecting ring closure in other N-phosphorylated compounds. A further application of the effect of molybdate on phosphocreatine is its use as a means of identi-

fying this compound in a mixture of such acid-molybdate-labile organophosphate compounds as are found in certain tissue extracts (Barker and Ennor, unpublished data).

TABLE 2

EFFECT OF AMMONIUM MOLYBDATE (FINAL CONC. = $1.25 \times 10^{-4}M$) AND VARYING pH UPON THE HYDROLYSIS OF PHOSPHOCREATINE

Conditions of hydrolysis: 30 min. at 37°C. Buffer, potassium hydrogen phthalate in a final conc. of 0.1M. The following abbreviations are used: CRINE—creatinine; CR—creatine; P—inorganic phosphate

Molybdate	pH	CRINE ($\mu g.$)	CR		Total CR ($\mu g.$)	P ($\mu g.$)	Molar Ratio P/CR	Hydro- lysis %
			Equiva- lent ($\mu g.$)	CR ($\mu g.$)				
Present	0.1N HCl	31.0	36.0	17.5	53.5	12.9	1.02	89
Absent		4.1	4.8	35.7	40.5	10.2	1.06	70
Present	2.8	22	25.5	25.0	50.5	12.5	1.05	86
Absent		3.3	3.8	37.8	41.6	10.4	1.06	71
Present	3.5	12.4	14.4	30.9	45.3	11.0	1.02	76
Absent		3.6	4.2	37.8	42.0	10.3	1.04	71
Present	4.0	7.9	9.2	33.5	42.7	10.4	1.03	71
Absent		2.9	3.4	34.6	38.0	9.0	1.00	62
Present	4.6	2.9	3.4	26.1	29.5	7.0	1.00	48
Absent		2.9	3.4	26.1	29.5	7.0	1.00	48
Present	5.4	2.3	2.7	10.7	13.4	3.2	1.01	22
Absent		2.3	2.7	10.7	13.4	3.2	1.01	22

V. ACKNOWLEDGMENTS

The authors wish to express thanks to the Director-General of Health and to the Director of the Commonwealth Serum Laboratories, Melbourne, for generous provision of laboratory space pending the establishment of this Department in Canberra. It is also a pleasure to thank Miss J. D'Alton and Mr. R. Frater for skilled technical assistance.

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INACTIVATION OF GONADOTROPHINS

III. INACTIVATION AND MODIFICATION OF SERUM GONADOTROPHIN BY PERIODATE IONS

By W. K. WHITTEN*

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Summary

Serum gonadotrophin was rapidly but not completely inactivated by dilute solutions of potassium periodate. The effect of time and of concentration of periodate on the reaction were studied. No evidence was obtained of an increase in activity after treatment with still greater dilutions of periodate.

The residual activity, after treatment with periodate, showed evidence of modification as it produced a greater degree of luteinization than comparable doses of normal hormone in both normal and hypophysectomized animals. In addition, it was no longer inactivated by influenza virus. No departure from parallelism was observed between the dose response curves of treated hormone and those produced by untreated gonadotrophin.

The activity of the oxidized hormone was not altered by subdivision of the dose, which indicated that the inactivation was not due to change which permitted excretion.

Unlike luteinizing hormone and chorionic gonadotrophin, the treated serum gonadotrophin produced significant growth of the ovaries of hypophysectomized rats.

I. INTRODUCTION

In the first paper of this series (Whitten 1948) it was shown that serum gonadotrophin was inactivated by preparations of influenza virus and receptor-destroying enzyme from *Vibrio cholerae* and it was concluded that the inactivation was enzymic. Other substrates of these enzymes have been shown to be highly susceptible to the action of dilute aqueous periodate. Hirst (1949) showed that this reagent inactivated the "receptor-substance" of red cells and McCrea (1948) found that "Francis inhibitor" was similarly affected. Comparable results were obtained by Burnet (1948) with cyst mucoid except that after treatment with low concentrations of periodate an increase in inhibitory titre, both with regard to infection and haemagglutination was observed when tested against one strain of virus.

In view of these findings and those of Pearse (1948), who demonstrated gonadotrophins histologically after periodic oxidation, it was decided to investigate the action of periodate on the biological properties of serum gonadotrophin.

The response elicited by serum gonadotrophin in the ovaries of normal immature female rats is mainly one of follicle stimulation but various degrees

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of luteinization of the *membrana granulosa* occur, particularly at high dose levels. The response in hypophysectomized animals is similar but luteinization is less in evidence (Noble *et al.* 1939; Rowlands and Williams 1940). The last two authors postulated the presence in serum gonadotrophins of two active components, follicle-stimulating which predominates, and luteinizing.

Evans *et al.* (1936) and Hellbaum (1937) claimed to have separated serum gonadotrophin into two fractions. Cole, Pencharz, and Goss (1940) were unable to confirm these findings nor could they detect any qualitative difference between crude and highly purified preparations. Li, Evans, and Wonder (1940) prepared serum gonadotrophin in an electrophoretically homogeneous form, the action of which was indistinguishable from that of the original serum. From these findings it was concluded that the activity of serum gonadotrophin was dependent on a single hormone. Nevertheless, Rimington (1946) was unable to arrive at the same conclusion since he separated from an electrophoretically homogeneous preparation a fraction which was about four times as active as the original material.

One of the preparations used in this study produced an unusually high degree of luteinization. Rowlands and Williams (*loc. cit.*) describe one, and probably two, similar preparations. It is perhaps unfortunate that one of these was used by Noble *et al.* (*loc. cit.*) in their description of the action of the hormone. These preparations indicate that the follicle-stimulating and luteinizing activities of serum gonadotrophin may be, to some extent, independent, and the evidence produced by Kupperman, Meyer, and McShan (1941) by the use of suitable antisera supports this view. However, Cartland and Nelson (1937) were unable to detect any alteration in the nature of the response to serum gonadotrophin after partial inactivation with formaldehyde.

As the action of periodate is relatively mild and selective, it was considered that a study of its reaction with serum gonadotrophin might yield some useful information regarding the hormone substance.

II. MATERIALS AND METHODS

Serum gonadotrophin.—Two preparations of serum gonadotrophin, Nos. 1 and 2, were supplied by Organon and assayed about 37 I.U. per mg. A third preparation, No. 3, was kindly supplied by Dr. C. W. Emmens of the Physiology Department, School of Veterinary Science, University of Sydney. This had been prepared by Professor C. Rimington several years previously and then assayed 2,000 I.U. per mg. It was assayed prior to use against the International Standard, at two dose levels and with separation of litter mates, when it contained 611 I.U. per mg. with 1 per cent. fiducial limits of 506 and 746 I.U. per mg. Histological examination of the ovaries of these groups showed that it produced an unusually high degree of luteinization. There was, however, no significant departure from parallelism in the dose response curves. All solutions of these hormones were prepared in distilled water before use.

Potassium periodate.—Potassium periodate of A.R. purity was prepared fresh and at the required concentration in M/15 phosphate buffer at pH 6.0.

Influenza virus.—Allantoic fluid of high titre from chick embryos which had been infected with LEE. B. influenza virus was kindly supplied by Mr. J. H. Whittam, of the Pathology Department, School of Veterinary Science, University of Sydney.

Hypophysectomized animals.—Female rats weighing 60-70 g. were hypophysectomized by the retropharyngeal approach under ether anaesthesia. Body weight records and examination of the *sella turcica* at death served as checks of the completeness of the operation. Injections were commenced 6-8 days after the operation.

Biological assay.—The gonadotrophin was assayed according to the method of the British Pharmacopoeia (1948) and the animals used were 23-25 day old female Wistar rats bred in this laboratory. The numbers of animals used, together with information regarding litter distribution and dose levels, are given in the tables. Preliminary observations were carried out and ovarian weight *per se* was used as the criterion of hormonal activity.

Experimental procedure.—Equal volumes of hormone and periodate solutions were mixed at room temperature and the reaction allowed to proceed for the specified time, when any excess periodate was reduced by the addition of glucose. The concentration of periodate is expressed as the molarity after mixing.

III. OBSERVATIONS

Preliminary tests showed that serum gonadotrophin was rapidly inactivated by M/1000 potassium periodate in M/15 phosphate buffer at pH 6.0, at room temperature. Previous reduction of the periodate by glucose prevented the inactivation as shown in Table 1.

TABLE 1
MEAN OVARIAN WEIGHTS OF GROUPS OF TEN RATS INJECTED WITH 25 I.U. OF SERUM GONADOTROPHIN (PREPARATION No. 1), UNTREATED, TREATED WITH PERIODATE, AND WITH REDUCED PERIODATE

Treatment	Mean Ovarian Wt. (mg. $\pm$ S.E.)
Untreated	68 $\pm$ 5
M/500 periodate	19 $\pm$ 1
M/500 periodate (reduced)	65 $\pm$ 4

(a) Effect of Concentration of Periodate

The concentration of periodate necessary to give maximum inactivation in 30 minutes of a solution of preparation No. 1 containing 25 I.U. per ml. was determined. The treated hormone was injected into groups of seven animals at a dose equivalent to 25 I.U. of the original hormone. The mean ovarian weights are given in Table 2, from which it is evident that no further significant inactivation occurs after the concentration of M/10,000 is reached. Similar results were obtained with preparation No. 2. There is no evidence from these figures of any increase in gonadotrophic activity corresponding to the increase of inhibitory titre observed by Burnet (*loc. cit.*) with cyst mucoid.

(b) Effect of Time on the Reaction

Serum gonadotrophin was permitted to react with periodate for 5, 10, 30, and 60 minutes, when the reaction was stopped by the addition of excess glucose. The reaction mixture contained 25 I.U. of serum gonadotrophin (No. 1) per ml. of M/1000 KIO₄. One ml. of these solutions was injected into each of the groups of six rats. The mean ovarian weights of these groups are

TABLE 2
MEAN OVARIAN WEIGHT OF GROUPS OF SEVEN RATS INJECTED WITH 25 I.U. OF SERUM GONADOTROPHIN (PREPARATION No. 1) AFTER TREATMENT WITH INCREASING CONCENTRATIONS OF PERIODATE

Concentration of Periodate	Mean Ovarian Wt. (mg. $\pm$ S.E.)
0	73 $\pm$ 6
M/40,000	60 $\pm$ 3
M/30,000	65 $\pm$ 8
M/20,000	46 $\pm$ 2
M/15,000	32 $\pm$ 3
M/10,000	21 $\pm$ 2
M/5,000	22 $\pm$ 2
M/1,000	18 $\pm$ 1
M/500	19 $\pm$ 1
Untreated animals	15 $\pm$ 1

given in Table 3. Similar results were obtained with the other hormone preparations. From these results it is evident that no significant reduction of hormone activity occurred after an interval of five minutes. Additional observations indicated that subsequent loss did occur but at a much slower rate.

TABLE 3
MEAN OVARIAN WEIGHTS OF GROUPS OF SIX RATS INJECTED WITH 25 I.U. OF SERUM GONADOTROPHIN AFTER EXPOSURE TO M/1000 PERIODATE FOR VARIOUS TIMES

Time (min.)	Mean Ovarian Wt. (mg. $\pm$ S.E.)
0	148 $\pm$ 7
5	34 $\pm$ 3
10	31 $\pm$ 2
30	25 $\pm$ 1
60	25 $\pm$ 2

The initial rapid inactivation resembles that observed by McCrea (loc. cit.) with "Francis inhibitor" and it is considered that this may be due to oxidation of the carbohydrate portion of the hormone, whereas the subsequent loss may correspond to the slow inactivation of the biological properties of proteins reported by Goebel, Olitsky, and Saenz (1948). However, no attempt was

made to determine the nature of the reaction or reactions involved. In all but one of the subsequent experiments the reaction was stopped by the addition of glucose after an arbitrary period of 30 minutes.

(c) *Residual Activity of Periodate-treated Serum Gonadotrophin*

By the use of the information described earlier, the amount of hormonal activity remaining after treatment with excess periodate for 30 minutes was determined for the three samples of gonadotrophin. The treated material was assayed against untreated hormone from the same sample. Unfortunately, the high doses required proved toxic with one preparation and no accurate estimate

TABLE 4
PERCENTAGE OF ACTIVITY OF SERUM GONADOTROPHIN LOST AFTER TREATMENT
WITH EXCESS PERIODATE FOR 30 MIN.

Preparation No.	No. of Rats per Dose	Loss (%)	Fiducial Limits of Error $P = 0.99$
1	10	95 ⁽¹⁾ approx.	
1	8 ⁽²⁾	96.4	94.9-97.4
2	9	93.1	91.6-94.8
2	9	93.0	85.6-99.2
3	8 ⁽³⁾	83.4	77.4-87.2

⁽¹⁾ Preparation toxic at high doses.

⁽²⁾ Litter mates segregated.

⁽³⁾ Four dose levels of treated and untreated hormone used.

of potency could be made. The results are given in Table 4 and a typical analysis of variance is given in Table 5. It is evident that preparations No. 1 and No. 2 retained 4-7 per cent. of their original activity whereas 17 per cent. of preparation No. 3 was recovered. This apparent difference may be due to the fact that considerable loss of activity had occurred before use in these experiments. However, if the residual activity is calculated on the potency of No. 3 when originally prepared (2000 I.U./mg.), the result, viz. 6.0 per cent., is in closer agreement with those obtained for Nos. 1 and 2. This will be further considered when the qualitative nature of the residual hormone is discussed.

An analogous small amount of residual activity was observed by Burnet (1949) when mucin was treated with periodate and tested against Newcastle Disease virus.

(d) *Nature of Residual Gonadotrophin*

(i) *Dose Response Curves.*—A complete examination of the dose response curve for periodate-treated gonadotrophin has not been made. However, in the assay of residual activity of preparation No. 3, four dose levels of treated and control hormone were administered to groups of eight rats. The results obtained are shown in Table 6. The group injected with untreated hormone at

the lowest dose and that receiving the highest dose of treated hormone were responsible for departure from linearity and were omitted from analysis. This analysis confirmed the absence of departure from parallelism observed in the previous assays.

TABLE 5
DATA AND ANALYSIS OF VARIANCE OF ASSAY OF TREATED SERUM GONADOTROPHIN
(PREPARATION No. 2) AGAINST UNTREATED HORMONE

Untreated		Treated	
Dose 16 I.U. Ovarian Wt. (mg.)	Dose 24 I.U. Ovarian Wt. (mg.)	Dose 267 I.U. Ovarian Wt. (mg.)	Dose 400 I.U. Ovarian Wt. (mg.)
67	79	57	93
52	59	74	128
56	109	47	116
43	122	77	128
52	110	72	91
41	76	50	149
45	114	79	100
71	78	75	118
38	80	38	94
Mean 51.7	91.9	63.2	113.0

ANALYSIS OF VARIANCE

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Ratio	P
Between samples	1	2401.0	2401.0	7.7	< 0.05
Slope	1	18225.0	18225.0	58.4	< 0.001
Departure from parallelism	1	205.4	205.4	—	—
Error	32	9976.4	311.8	—	—
Total	35	308.08			

(ii) *Histological Response in Normal Animals.*—Ovaries from the groups of animals used in the assays were examined after sectioning and staining with haematoxylin and eosin. An estimate of the degree of luteinization ranging from one to five was allotted. The results obtained for preparation No. 2 are given in Table 7. Analysis revealed that a significantly greater degree of luteinization ($P < 0.001$) occurred in the ovaries of rats injected with oxidized hormone. In addition, no significant difference was detected between the nature of the response of the two doses of the treated hormone, whereas, with the untreated gonadotrophin the higher dose produced a significantly greater degree of luteinization ($P < 0.001$). This latter finding confirms the accepted increase in luteinization which occurs with increased doses of normal serum gonadotrophin. Plate 1, Figures 1 and 2, shows typical ovaries from animals injected with treated and untreated hormone. Similar results were obtained with preparation No. 1. With preparation No. 3, as indicated earlier, a high

degree of luteinization was observed with the untreated hormone—however, all follicles of animals injected with this oxidized hormone were completely luteinized.

TABLE 6
DATA OF ASSAY OF TREATED AGAINST UNTREATED SERUM GONADOTROPHIN
(PREPARATION No. 3) AT FOUR DOSE LEVELS

		Untreated				Treated			
Dose (I.U.)	5	10	20	40	70	140	280	560	
	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	
	27	44	108	150	56	102	237	204	
	30	54	110	174	45	100	182	253	
	30	52	92	141	71	56	143	219	
	25	34	80	104	63	93	157	251	
	29	62	65	215	56	90	140	228	
	30	30	58	160	57	118	192	226	
	25	26	99	124	65	76	154	218	
	33	44	90	140	59	80	148	178	
Mean	28.6	43.3	87.8	151.0	51.8	90.5	168.0	222.1	

The high degree of luteinization prior to treatment suggests that changes similar to those produced by periodate have already occurred in preparation No. 3. This evidence supports the calculation made in Section III (*c*) of the residual activity using as a basis the original activity before storage.

TABLE 7
DATA OF GRADING OF HISTOLOGICAL NATURE OF RESPONSE TO TREATED AND UNTREATED
SERUM GONADOTROPHIN (PREPARATION No. 2)

Dose (I.U.)	Treated		Untreated	
	267	400	16	24
Mean ovarian wt. (mg.)*	63.2	113.0	51.7	91.9
Histological Grading				
Mean	4.8	4.8	1.5	2.9
Range	4-5	4-5	1-3	2-3

* See Table 5.

(iii) *Response in Hypophysectomized Animals.*—The response produced by two comparable doses of both treated and untreated hormone (preparation No. 1) was examined. The doses and results are given in Table 8. Analysis revealed no significant difference between the response to the two samples, at the selected dose rates, a significant slope ($P < 0.01$) for the combined response curve, and no departure from parallelism of the response curves of the samples.

On section it was seen that the untreated hormone produced almost pure follicle stimulation. The ovaries of the animals injected with the treated hormone showed similar evidence of follicular growth but the *membrana granulosa* of almost all follicles with atria was partly or completely luteinized. Plate 1, Figures 3 and 4, shows typical responses to these substances in litter mates, the ovaries of which weighed 50 and 52 mg. respectively. These findings confirm those observed in intact animals and show that the altered response is not mediated through the anterior pituitary.

TABLE 8
RESPONSE IN HYPOPHYSECTOMIZED RATS TREATED WITH NORMAL AND OXIDIZED
SERUM GONADOTROPHIN (PREPARATION No. 1) AT TWO COMPARABLE DOSES

Dose (mg.)	Normal Gonadotrophin		Oxidized Gonadotrophin	
	0.5 Ovarian Wt. (mg.)	1.0 Ovarian Wt. (mg.)	10.0 Ovarian Wt. (mg.)	20.0 Ovarian Wt. (mg.)
	56	51	33	43
	48	42	37	42
	55	61	16	60
	43	66	28	57
	45	40	44	87
	33	46	42	50
	35	83	39	41
	36	52	44	102
	29	57	28	42
	20	28	50	48
	48	23	26	28
	33	46		23
				46
Mean	40.1	49.6	35.2	51.5

A similar modification of the response to serum gonadotrophin in hypophysectomized animals treated with oestrogens post-operatively has been reported by Williams (1945). In a limited number of animals the author has not observed as great a degree of luteinization by this procedure as with the oxidized hormone. Nevertheless, it is possible that endogenous oestrogen may be responsible for the increased luteinization.

(iv) *Effect of Subdivision of the Dose.*—Since serum gonadotrophin is not excreted and thus produces a maximum response following a single injection, it was considered that the reduction in activity produced by periodate may have resulted from an alteration which permitted excretion. In order to examine this possibility, two groups of ten animals were injected with equal quantities of treated hormone. One group received the total dose on the first day, whereas the other was injected with one-fifth the dose for five successive days. Both groups were killed on the sixth day, and the ovaries removed, fixed, and weighed. The mean ovarian weight of the group which received the single

injection was 91.0 mg. whereas that of the other group was 61.8 mg., the difference being significant ($P < 0.01$). Thus subdivision of the dose did not increase the response.

(v) *Effect of Influenza Virus*.—Anderson (1947) has shown that cells treated with periodate are altered with respect to their reaction with influenza virus, so it was decided to examine the effect of the virus on treated gonadotrophin. Two samples of periodate-treated gonadotrophin (preparation No. 1) were incubated with active influenza virus and assayed against duplicate aliquots which had been incubated with inactivated virus. The results in Table 9 show that no loss occurred. A qualitative test carried out concurrently showed that the action of influenza virus on intact gonadotrophin was not inhibited by the presence of an equivalent concentration of glucose-reduced periodate.

TABLE 9
PERCENTAGE OF ACTIVITY OF OXIDIZED GONADOTROPHIN RECOVERED AFTER
TREATMENT WITH INFLUENZA VIRUS

Preparation No.	No. of Rats per Dose	Per cent. Recovered	Fiducial Limits of Error $P = 0.99$
1	10	101.7	70.4-148.9
1	7*	107.8	66.5-159.7

* Litter mates segregated.

IV. DISCUSSION

As there has been no substantial evidence of the separation of serum gonadotrophin into follicle-stimulating and luteinizing components it has been accepted generally that this hormone is a single mucoprotein or a mucoprotein complex, which exhibits a combination of both actions. This dual nature of the hormone has been used as an argument against the concept of two anterior pituitary hormones. In this regard, it is of interest that treatment of serum gonadotrophin with periodate ions modifies it so that luteinization becomes more evident or even predominates.

The most obvious explanation of the inactivation and modification of serum gonadotrophin by periodate is that the follicle-stimulating component is reduced whereas that responsible for luteinization is relatively unaltered. The present assay methods do not permit determination of the absolute amounts of those components so it is impossible to verify this assumption. If, however, it is correct, it indicates that follicle stimulation depends on groupings susceptible to periodic oxidation. The conditions under which the modification occurred and the mucoid nature of the hormone suggest that these are the carbohydrate groups of the hormone.

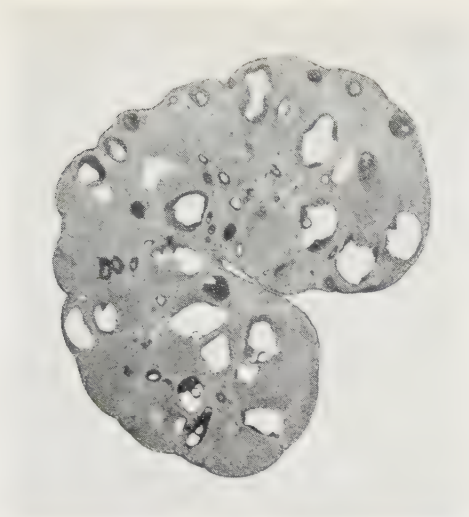


Fig. 1



Fig. 2



Fig. 3

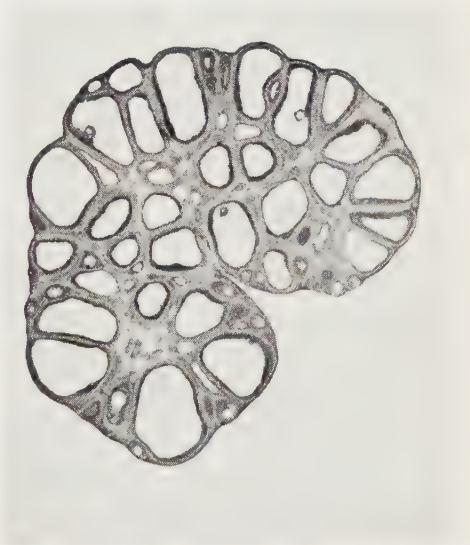


Fig. 4

Fig. 1.—Photomicrograph of section of ovary of immature rat injected with 16 I.U. of serum gonadotrophin (preparation No. 2). $\times 16$. Ovarian wt. 52 mg.

Fig. 2.—Photomicrograph of section of ovary of immature rat injected with 267 I.U. of serum gonadotrophin (preparation No. 2) treated with periodate ions. $\times 16$. Ovarian wt. 57 mg.

Fig. 3.—Photomicrograph of section of ovary of hypophysectomized rat injected with 1 mg. of serum gonadotrophin (preparation No. 1). $\times 16$. Ovarian wt. 52 mg.

Fig. 4.—Photomicrograph of section of ovary of hypophysectomized rat injected with 20 mg. of serum gonadotrophin (preparation No. 1) treated with periodate ions. $\times 16$. Ovarian wt. 50 mg. The animals used to obtain Figures 3 and 4 were litter mates.

V. ACKNOWLEDGMENTS

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THE CHEMICAL MECHANISM OF THE OXIDATIVE DEAMINATION OF AMINO ACIDS BY CATECHOL AND POLYPHENOLASE

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Summary

The continuous deamination of certain amino acids by the system catechol-polyphenolase takes place through intensely coloured intermediate compounds. These intermediates are formed by the combination of equimolecular proportions of *o*-quinone with amino acids or secondary amines. They are presented as structural homologues of adrenochrome or hallachrome and probably of Szent-Györgyi's tyrin (1925).

It is suggested that the continuous deamination is brought about through condensation of the colour compound with one further molecule of a suitable amino acid and rearrangement and hydrolysis into keto acid and substituted amino phenol. It is supposed that the latter is re-oxidized with liberation of ammonia and regeneration of the *o*-quinonoid colour compound. A formulation of the chemical mechanism is tentatively proposed and individual steps of the reaction are compared with observations made in the oxidation of amino acids by other polyketo compounds like alloxan and isatin. The conditions and limitations of the reaction are discussed.

I. INTRODUCTION

Szent-Györgyi (1925) characterized a red substance "tyrin" claimed to be widely distributed in the animal and vegetable kingdom and to act as a non-enzymic hydrogen carrier in the sense of Palladin's group of respiratory pigments. The oxidative deamination of certain amino acids by the action of tyrosinase in the presence of catechol was demonstrated by Robinson and McCance (1925) and Happold and Raper (1925). The latter suggested that the reaction was due to the formation of *o*-quinone which combined with the amino acid, leading to the final decomposition of the complex with oxidation of the amino acid and formation of deeply coloured substances. Platt and Wormall (1927) showed that the formation of coloured compounds with the carrier action of tyrin could be brought about by the interaction of catechol, polyphenolase, and amino acids and "that amino-acids . . . are solely responsible for the red pigment formation with . . . the catechol-oxidase system."

The characteristics given by Szent-Györgyi for tyrin are identical with those observed for the colour compounds formed from catechol and amino acids: the compounds are intensely red to brown-red, soluble in water and alcohols, but insoluble in ether; they can be adsorbed on charcoal, but are

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difficult to elute without decomposition. They are moderately stable but prolonged standing or attempts at purification, even marked pH changes, lead to discoloration; depending on the conditions, brown suspensions or black melanin-like precipitates are formed. Reducing agents (nascent hydrogen, sulphite, certain amino acids) reduce them to leuco compounds which are readily re-oxidized by atmospheric oxygen. This redox system was further examined by James *et al.* (1948) and James and Beevers (1948) during their investigation of a polyphenolase prepared from *Atropa belladonna*. It was found that the colour compound is formed from equimolecular proportions of catechol and secondary amines or primary or secondary amino acids—but not from ammonia or primary or tertiary amines—and that no oxidation of the amino acid takes place with these proportions; but that any of these colour compounds acts as a catalyst in the oxidation of a suitable amino acid added in excess to the system.

With the belladonna enzyme no liberation of carbon dioxide took place; the ammonia liberated was quantitatively determined and was found to correspond to the excess oxygen uptake over that required for the oxidation of catechol alone (two atoms for one molecule of catechol); the keto acid could be identified in the oxidation products from glycine and alanine. The authors proposed for the colour compound the structure of a *p*-amino-*o*-quinone and related this structure to that of adrenochrome and dopa-quinone which show an identical, though weaker carrier action (Fig. 1). Jackson and Kendal (1949) investigated the purple colour compound formed when crude extracts of mushrooms oxidize catechol and identified it as consisting of equimolecular proportions of hydroxyproline and catechol according to the formula in Figure 1; their observations and conclusions confirm the results obtained by James *et al.* (1948).

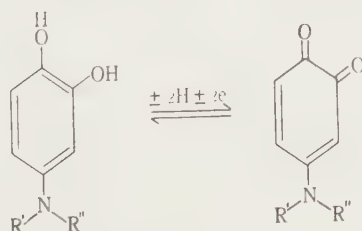


Fig. 1.—Leuco and oxy form of the colour compound.

The present work, begun at the Department of Botany, Oxford, in 1947 and continued by one of the authors (E.M.T.) at the Department of Physiology, University of Melbourne, is mainly concerned with the chemical mechanism of amino acid oxidation by the quinonoid redox system. It is intended (1) to discuss the formation and nature of the coloured compounds in more detail, (2) tentatively to present a formulation of the chemical mechanism of continuous amino acid oxidation, (3) to explain why no oxidation occurs with

equimolecular proportions of catechol and amino acids and why secondary amines or amino acids are not oxidized by the system, and (4) to consider the conditions and limitations of the reaction.

II. MATERIALS AND METHODS

The substances used were of Analar or reagent quality; no indications were found that any of them contained impurities interfering with the reactions studied. The solutions were freshly prepared before each experiment. Conditions and concentrations were chosen so as to conform with those suitable for enzymic reaction: catechol from M/500 to M/1000 and amino acids or amines, if added, in molar proportions; pH between 6 and 7.8 (at higher pH entirely different reactions occur); temperature between room temperature and 37.7°C. *o*-Quinone was prepared by the method of Willstätter and Müller (1911), adrenochrome by the method of Randall (1946), and 1,2,4-trihydroxybenzene by the method of Jackson (1939).

Enzyme preparation.—In the earlier part of the work the belladonna enzyme used by James *et al.* (1948) was employed; the graphs presented in the present paper were obtained by using a polyphenolase prepared from the related plant *Duboisia myoporoides* R.Br. by the same procedure: about 50 g. of *Duboisia* leaves are ground in a Waring Blendor for 3-5 minutes with 75 ml. of cold acetone, filtered, and the residue re-extracted twice with the same volume of acetone; the last extract shows only a slight green colour. The residue is then extracted three times with 30 ml. of distilled water; the last extract should give only a faint reaction for amino acids with ninhydrin and no catechol or catechin reaction with ferric chloride. The residue is again washed with acetone once and dried in a desiccator under reduced pressure; it consists of a pale whitish or brownish powder; yield: 7-8 per cent. of the weight of the fresh leaves. Kept in a desiccator the preparation is stable for several weeks; 25 mg. of it are sufficient to guarantee the oxidation of 1 ml. M/100 catechol or adrenaline without risk of inactivation. Since the enzyme preparation is insoluble in water, the reaction products can be obtained protein-free by filtration. Figure 2 shows the oxygen uptake of the catechol-polyphenolase system alone and with varying amounts of glycine in phosphate buffer, pH 7.8 at 30°C.

Some preparations gave a practically linear oxygen uptake over four to five molecules of amino acid within three to four hours of the manometric experiment; with others the rate of oxidation dropped quickly. It appeared that the *Duboisia* enzyme used in later work was more constant in its action than the belladonna enzyme with which the authors worked in Oxford.

Oxygen uptakes were measured in Warburg apparatus under the conditions mentioned; ammonia and volatile amine in the reaction products were separated by the method of Conway (1947). Colorimetric measurements were made with a Lumitron colorimeter (Photovolt, New York).

III. RESULTS

(a) Colour Compounds

(i) *Formation of the Compounds under Varying Conditions.*—Catechol alone does not form coloured compounds with ammonia, amines, or amino acids under the conditions and dilutions of the enzymic reaction. On addition of oxidizing agents (ferricyanide, hypochlorite, chloramine T, etc.) colour formation with amino acids or secondary amines proceeds rapidly at room temperature, faster at slightly alkaline than at more acid pH; ammonia, methyamine, and tertiary amines give only slowly a brown, turbid discoloration.

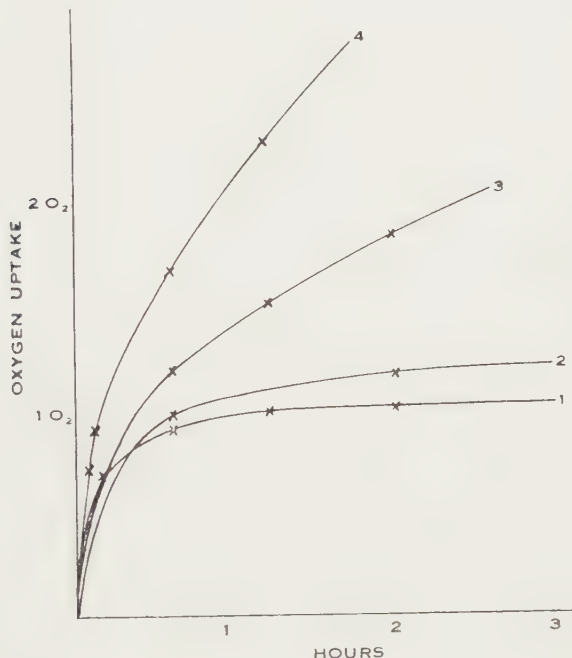


Fig. 2.—Oxygen uptake of catechol alone and with varying excess of glycine. Warburg; phosphate buffer pH 7.8; 30°C.; 100 shaking; 1 ml. M/100 catechol in final concentration of M/500. 25 mg. *Duboisia* enzyme.

Curve 1. Catechol alone.

Curve 2: Catechol plus 5 molar equivalents glycine.

Curve 3: Catechol plus 20 molar equivalents glycine.

Curve 4: Catechol plus 40 molar equivalents glycine.

The colour compounds formed are identical with those formed in the enzymic reaction and can substitute for them in the deamination of suitable amino acids (James *et al.* 1948; Jackson and Kendal 1949). With primary amino acids red-brown colours are formed. The beetroot purple formed with secondary amines and amino acids (proline, sarcosine, etc.) is distinctive and can serve as a qualitative test for the group or for the presence of a polyphenolase.

(ii) *Steps of the Reaction*.—If a dilute solution of catechol is shaken for one to two minutes with excess of the insoluble enzyme preparation and filtered, a green solution is obtained which turns quickly reddish and turbid brown. On addition of amino acid or a secondary amine to the green filtrate the characteristic colours are formed instantaneously, suggesting that the amino compounds combine non-enzymically with an oxidation product of catechol.

(iii) *o-Quinone as Intermediate*.—The addition of a green ethereal solution of *o*-quinone to water leads to a greenish aqueous solution which turns gradually reddish and turbid brown, exactly as does the filtrate from a catechol-enzyme mixture during the period of rapid oxygen uptake. If ethereal *o*-quinone is added to a dilute solution of amino acids or secondary amine, the colour compounds are formed. The solutions are as stable as those formed in the enzymic reaction.

The reaction can be further differentiated. If to an aqueous solution of an amino acid or a secondary amine, only a small amount of ethereal green *o*-quinone is added, the initial weak colour fades quickly to a brownish tint. On strong shaking with air the typical colour is formed to its full extent. With excess of *o*-quinone it is formed immediately.

The presence of the aqueous medium is, however, not essential; the colour compounds are formed equally well if ethereal *o*-quinone is added to an alcoholic solution of proline or a secondary amine or if water-free alcoholic solutions of catechol and amino compound are directly oxidized by silver oxide. Using this procedure Jackson and Kendal (1949) synthesized the compound formed from equimolecular proportions of homocatechol and hydroxyproline-ester; the compounds from amino acids were formed but could not be separated in a pure state. The present authors obtained the dimethylamine compound in clusters of small, black needles on adding ether to the reaction mixture and cooling on dry ice; the crystals decomposed, however, before the temperature could be raised to 0°C. Attempts at the syntheses are being continued. It is inadvisable to attempt the oxidation of catechol by silver oxide in the presence of undissolved or concentrated amino acids or other reducing substances; the reaction is likely to assume explosive violence.

(iv) *1,2,4-Trihydroxybenzene as Possible Intermediate*.—*o*-Quinone in aqueous solution is supposed to be quickly hydrated to 1,2,4-trihydroxybenzene (hydroxyquinol); it was at first considered that the colour compounds may be formed by combination of the amine with the latter (James *et al.* 1948). Crystalline 1,2,4-trihydroxybenzene was, in a series of experiments, substituted for catechol in enzymic as well as in non-enzymic reactions under otherwise identical conditions. In all cases strong oxidation took place and oxidizable amino acid, if present, was oxidized, but it was not possible to obtain or demonstrate the typical colour compounds. The solutions assumed an intense dark red colour, unlike and much stronger than the colours produced from identical concentrations of catechol and amino acid. The same colour is formed in the absence of amino compounds; it is due to the semiquinone

synthesized from equimolecular proportions of 1,2,4-trihydroxybenzene and 2-hydroxy-*p*-quinone by Willstätter and Müller (1911). This colour, however, is *not* observed if catechol is oxidized by ferricyanide or by the enzyme; the solutions turn yellow or orange with a low intensity of colour and finally turbid brown. It was not possible to duplicate the result of the oxidation of catechol alone or in the presence of amino compounds by substituting 1,2,4-trihydroxybenzene for catechol.

(v) *The Mono-Substituted Colour Compound* (Fig. 1).—The observations recorded in the previous work with belladonna enzyme (James *et al.* 1948) were confirmed in the present work using a polyphenolase from the leaves of *Duboisia myoporoides* (Solanaceae).

(vi) *Effect of Excess Amino Compound on Colour Intensity*.—In the enzymic oxidation of an equimolecular mixture of catechol and a secondary amine the colour compound is formed with the uptake of two atoms of oxygen for one molecule of catechol. If further amounts of secondary amine are added to the reaction mixture, no further oxygen uptake occurs but a considerable *intensification of the colour* is observed. Its degree is difficult to ascertain in the enzymic reaction where part of the colour compound is adsorbed on the enzyme preparation. If, however, the experiment is performed by adding, to the mixture of catechol and varying molar proportions of secondary amine, ferricyanide corresponding to the oxygen uptake in the enzymic reaction—four equivalents to one of catechol—it is seen that the colour intensity rises with increasing proportions of the amino acid and reaches a distinct *maximum with the proportions of two equivalents of amine to one of catechol*. Figure 3 shows the intensity of the purple colour formed with proline after $\frac{1}{2}$, 1, and 5 minutes; the blank value for catechol and ferricyanide alone, a faint yellow, is small. At room temperature the final intensity of the colour is reached within 5-10 minutes; on prolonged standing the solutions become turbid and discoloured. Hydroxyproline, sarcosine, and simple aliphatic secondary amines give similar curves.

The curves of the colour intensity seem to show a slight but constant kink at equimolecular proportions; up to this proportion they ascend steeply, almost linearly, above it they rise in a less steep, but again almost straight, line up to the proportions of two molar equivalents of secondary amine to one of catechol when they flatten out fairly abruptly.

The curves of the colour intensity formed in mixtures of catechol with varying molar proportions of primary amino acids, oxidizable or not oxidizable by the system, do not show the distinctive shape just described. The slope up to equimolecular proportions and slightly beyond is regular and there is possibly just beyond the equimolecular proportion a slight decrease in the rate at which the colour intensity increases, but there is no indication of a maximum at base : catechol proportions of 2:1. A maximum is only slowly reached with five to seven molar equivalents of amino acid (Figs. 3 and 4).

It will be noted that there is no difference between the shape of the curves formed from readily oxidizable amino acids (glycine) and those from acids which are oxidized only very slowly or not at all (glutamic acid); in view of the slow rate of amino acid oxidation no essential difference can be expected within the time of observation.

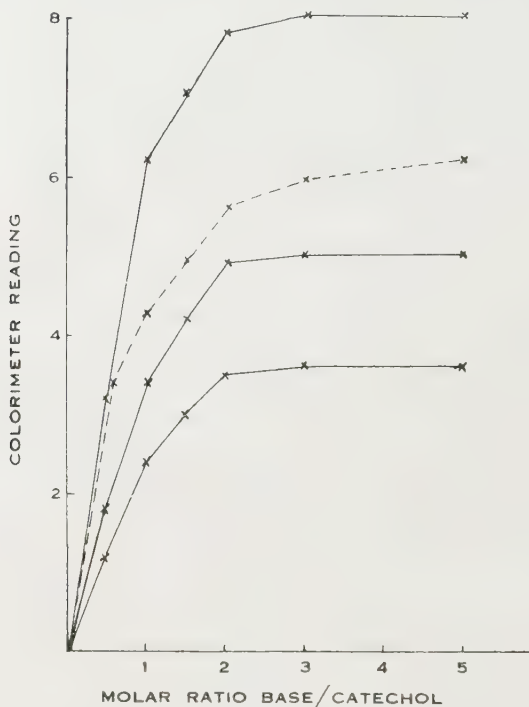


Fig. 3.—Optical density of the purple colour formed from catechol and varying proportions of proline within $\frac{1}{2}$, 1, and 5 min. Catechol concentration M/1000; oxidizing agent: ferricyanide, 4 mols. to 1 of catechol, added to the mixture of catechol and proline in M/15 phosphate buffer, pH 7.8, at room temperature. The broken line indicates the optical density of the red colour formed within 5 min. from catechol and glycine under the same conditions.

(vii) *Stability of the Colour Compounds*.—Since the reaction products obtained with one and two equivalents of amine—referred to as the mono- and di-substituted colour compound—could not be separated in a pure state, it was attempted to gain further information by comparing under varying conditions the stability of the compounds formed with volatile amines. Solutions of the compounds were prepared by reacting catechol with one and two molar equivalents of dimethylamine hydrochloride and enzyme until no further oxygen was taken up ($1\frac{1}{2}$ -2 hours) and filtering from the insoluble enzyme. Aliquots were

placed under varying conditions into the annulus of a Conway unit containing boric acid mixture (Conway 1947) in the centre well. The dimethylamine which diffused into the centre well was determined by titration with M/50

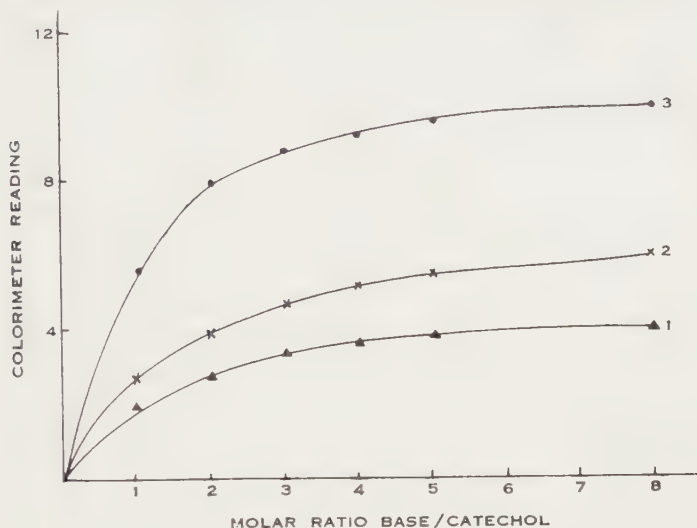


Fig. 4.—Optical density of the colour formed from catechol and glutamic acid (bottom), alanine (middle), and tyrosine (top) under the same conditions as given for Figure 3.

acid and identified by the purple colour formed with catechol and ferricyanide or enzyme. Table 1 gives the results of some experiments.

TABLE 1
VOLATILE AMINE DIFFUSED FROM THE MONO- AND DI-SUBSTITUTED DIMETHYLAMINO COMPOUNDS

Solution Added	Colour Compound	
	Mono-substituted Diffused	Di-substituted Diffused
M/10 Phosphate buffer to pH 7.8	0.02 ml. M/100	0.03 ml. M/100
M/10 Phosphate buffer to pH 8.5	0.022 ml. M/100	0.027 ml. M/100
M/10 Disodium phosphate to pH 9.4	0.023 ml. M/100	0.035 ml. M/100
M/100 Potassium carbonate to pH 10.5	0.15 ml. M/100	0.310 ml. M/100
0.5 ml. Conc. potassium carbonate to pH 11.4	0.528 ml. M/100	1.320 ml. M/100
1 ml. Conc. potassium carbonate to pH 11.4	0.642 ml. M/100	1.581 ml. M/100

Conway units containing 1 ml. M/100 colour compound in 5 ml. solution in the annulus and 2 ml. boric acid mixture in the centre well; 24 hours; room temperature. Blank values of solutions 0.015-0.030.

The results have to be interpreted with reserve, since all mono- and di-substituted colour compounds, including adrenochrome, undergo at more alkaline pH further oxidation with liberation of volatile amine. At weakly

alkaline pH and room temperature, however, the diffusion rate of dimethylamine is low and even then some oxidative decomposition cannot be excluded. The constant results are: *up to a pH of 9 to 9.5 both colour compounds are equally stable*; the amounts of dimethylamine diffused within 24 hours are within or near the blank values. Above a pH of 10 the di-substituted compound releases under identical conditions considerably higher amounts, up to one equivalent more, of dimethylamine than the mono-substituted compound, suggesting that the second molecule of dimethylamine is less firmly held than the first.

(viii) *Displacement of the Volatile Amine by Non-Volatile Amino Acid.*—

If a non-oxidizable amino acid, e.g. glutamic acid, is added to either the mono- or the di-substituted compound, not much change is observed: no oxygen is taken up and very little, if any, volatile amine is released; the solutions retain their purple colour. If a readily oxidizable amino acid like glycine is added, it is oxidized and so much ammonia is formed that it is not possible convincingly to demonstrate the release of volatile secondary amine. The colour of the solution, however, changes slowly from the *purple* tint of the compounds formed with secondary amines to the *brown-red* of the compounds formed with primary amino acids. If a slowly reacting amino acid, like alanine or isoleucine is added, the colour of the solution changes likewise to a more brownish tint, but only little oxidation takes place. If the experiment is conducted in a Conway unit at a pH between 8 and 9, it is found that only very little amine diffuses into the centre well from either the mono- or di-substituted compound. If the contents of the centre well are neutralized and added to a catechol-enzyme mixture, the purple colour formed with dimethylamine is developed clearly in the liquid from the di-substituted compound, indistinctly or not at all in the contents from the mono-substituted compound. The colour change from purple to red-brown is more convincing than the diffusion of dimethylamine under conditions which exclude the alkaline decomposition of the colour compounds.

(b) *Amino Acid Oxidation*

The observations recorded by James *et al.* (1948) were confirmed and need not be detailed. Figure 2 presents the results of a typical experiment.

(i) *Influence of pH.*—Nelson and Dawson (1944) state that at more acid pH the rate of formation of 1,2,4-trihydroxybenzene from *o*-quinone is markedly delayed, suggesting possibly a greater stability of *o*-quinone at the lower pH. It was found that the rate of catechol oxidation by the *Duboisia* enzyme at acid pH (5.5 to 6.5) is slightly lowered, the formation of the colour compounds, especially of those formed from primary amino acids, appears to be delayed and the rate of oxidation of an oxidizable acid is considerably slower. Experiments were conducted in which the reaction was started at acid pH and later on continued at a higher pH. Figure 5 (curves 1 and 2) shows the oxygen uptakes of catechol alone at pH 5.5 and the effect of the addition of 10 equivalents of glycine in a phosphate buffer of pH 8 after 1½ hours. Curves 3 and 4

show the oxygen uptakes of catechol plus 10 equivalents of glycine at pH 5.5 and the effect of the addition of 1 ml. phosphate buffer of pH 8 after 3 hours. It is seen that the oxygen uptakes increased immediately to about the same level as could be expected if the reaction had been started at the higher pH. Only the rate and not the course of the reaction appears to be affected by the lower pH. The big increase effected on alkalization after as much as three hours is remarkable.

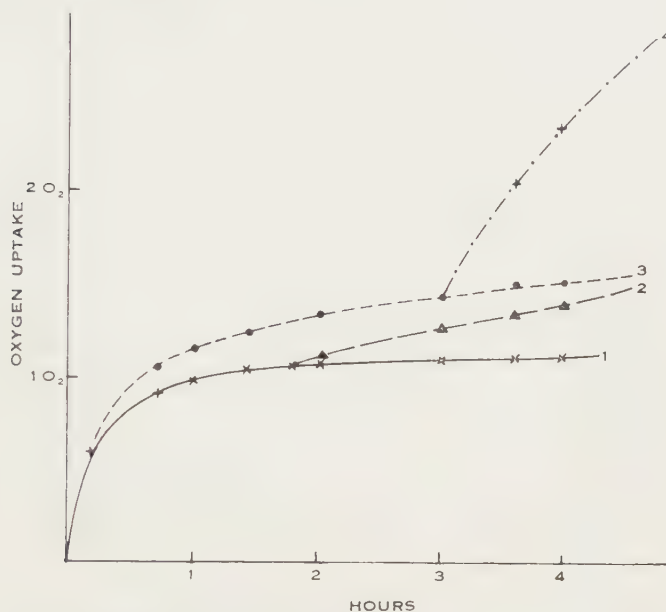


Fig. 5.—Effect of pH on the oxidation of glycine by the catechol-oxidase system. Warburg, 37°C.; 100 shaking; 30 mg. *Duboisia* enzyme; 1 ml. M/100 catechol in final concentration of M/500; initial pH 5.5; M/150 phosphate buffer.

Curve 1: Catechol alone, pH 5.5.

Curve 2: Catechol alone, pH 5.5; after 1 hr. 40 min. addition of 1 ml. M/10 glycine in M/10 phosphate buffer of pH 8.

Curve 3: Catechol plus 1 ml. M/10 glycine, pH 5.5.

Curve 4: Catechol plus 1 ml. M/10 glycine, pH 5.5; after 3 hr. addition of 1 ml. M/10 phosphate buffer of pH 8.

(ii) *Delayed Addition of an Oxidizable Amino Acid to the Catechol-Oxidase System.*—Figure 6 shows, at pH 7.8, the effect of delayed addition of 10 equivalents of glycine to the reaction mixture of catechol and enzyme at 30°C. The rate of additional oxygen uptake due to amino acid oxidation *decreased considerably on later addition* of the acid. The intensity of the colour formed decreased at the same rate as the faculty of the system to oxidize the amino acid. Addition of glycine after full oxidation of the catechol, 1½ to 2 hours, produced practically no colour and no additional oxidation. The two

functions appear to be connected. (From the graphs in Figure 5 it will be seen that the stability of the oxidizing system was greater at more acid pH.)

The solutions were examined and it was found that the *quinone reactions* (blueing of guaiacum, liberation of iodine from hydriodic acid) were strongly given by the filtrate from the insoluble enzyme during the period of intensive oxygen uptake, during the first half hour. Then they weakened; the final solutions after 1½ to 2 hours no longer gave any quinone reactions. *The faculty of oxidizing hydriodic acid disappeared parallel with that of forming the colour compounds and that of oxidizing additional amino acid.*

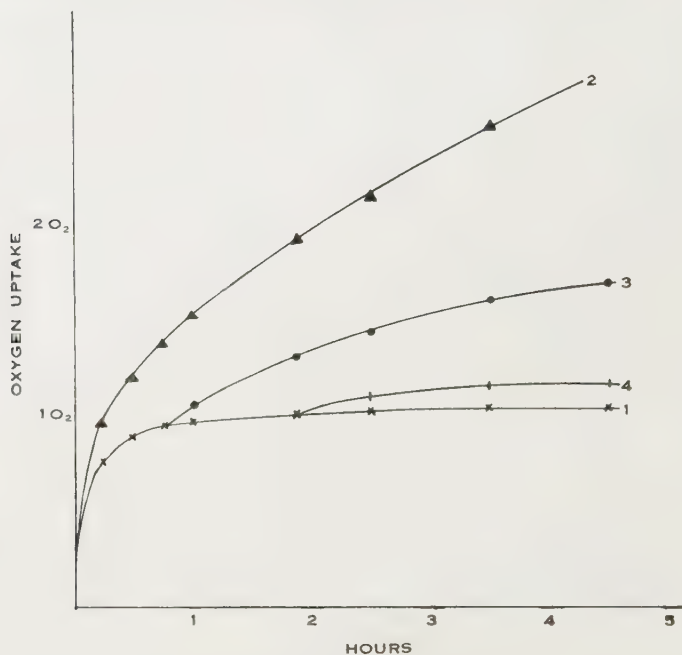


Fig. 6.—Effect of delayed addition of glycine to the catechol-oxidase system. Warburg, 30°C.; M/15 phosphate buffer pH 7.8; 100 shaking; 30 mg. *Duboisia* enzyme; 1 ml. M/100 catechol in final concentration of M/500; 1 ml. M/10 glycine.

Curve 1: Catechol alone.

Curve 2: Glycine added at the start.

Curve 3: Glycine added after 45 min.

Curve 4: Glycine added after 2 hr.

(iii) *The Reactivity of the Colour Compounds.*—In parallel experiments catechol and enzyme were allowed to react alone and in the presence of equimolecular proportions of amino acids or secondary amines to form the mono-substituted colour compounds; excess of glycine was added when the final oxidation level—two atoms of oxygen to one molecule of catechol—was reached. While the mixture containing catechol and enzyme alone showed little or no additional oxygen uptake and little or no development of colour, the flasks

containing the red compound from catechol and primary amino acids showed a considerable continuous oxidation, the rate of which was still exceeded by those containing the purple compound formed with secondary amines. (Figs. 7 and 8.) The same result was obtained when the addition of glycine was

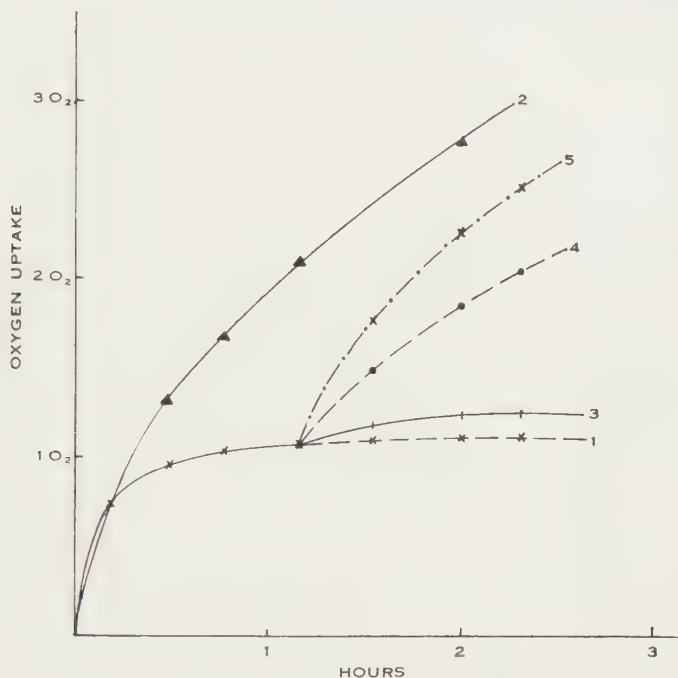


Fig. 7.—Effect of the addition of glycine to the reaction product of catechol alone and of catechol with equimolecular proportions of glycine and dimethylamine. Warburg, 37°C.; M/15 phosphate buffer pH 7.8; 100 shaking; 30 mg. *Duboisia* enzyme. 1 ml. M/100 catechol in final concentration of M/500. 1 ml. M/10 glycine was added to Expt. 2 at the start and to Expts. 3, 4, and 5 after 75 min.

Curve 1: Catechol alone.

Curve 2: Catechol alone.

Curve 3: Catechol alone.

Curve 4: 1 ml. M/100 catechol plus 1 ml. M/100 glycine.

Curve 5: 1 ml. M/100 catechol plus 1 ml. M/100 dimethylamine hydrochloride.

effected at an earlier or later stage of the experiment. Once formed, the mono-substituted colour compounds did not lose their power of amino acid oxidation to the degree to which the catechol oxidation products lost it.

The guaiacum reaction is unconvincing with strongly coloured solutions and even the hydriodic acid reaction presents difficulties, as the liberated iodine tends to undergo side reactions; if chloroform is overlain with a solution of potassium iodide acidified with sulphuric acid and the coloured solution is added with slight agitation, the liberation of iodine is unmistakable. It is

strongly shown by the amino acid compounds even after a few hours; the dimethylamine or proline compounds may show it after as much as 24 hours of standing at room temperature; adrenochrome shows it strongly in fresh solutions of the crystalline compound, but the reaction weakens on standing before the solutions become visibly turbid or discoloured.

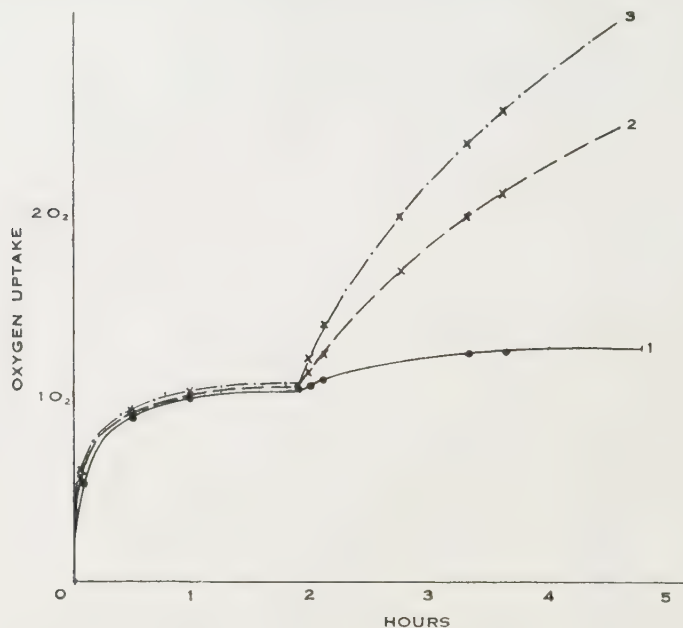


Fig. 8.—As for Figure 7 but with initial oxidation in M/100 phosphate buffer of pH 5.5. After 2 hr. 1 ml. M/10 glycine in M/15 phosphate buffer of pH 8.0 was added.

Curve 1: 1 ml. M/100 catechol.

Curve 2: 1 ml. M/100 catechol plus 1 ml. M/100 glycine.

Curve 3: 1 ml. M/100 catechol plus 2 ml. M/100 glycine.

(iv) *Effect of Secondary Amines on the Oxidation of Primary Amino Acids.*—The presence of dimethylamine or proline in moderate proportions does not lower the oxygen uptakes or oxidation rates of glycine; on the contrary it increases them. This effect is well developed already if catechol and secondary amines are present in equimolecular proportions; it is more marked if twice the amount of secondary amine is present, but still more with greater excess. There is no distinct optimal proportion of the secondary amine to catechol or to the amino acid. Figure 9 gives the result of an experiment in which a mixture of catechol with 10 equivalents of glycine was reacted alone and in the presence of 1, 2, 4, 20, and 40 equivalents of dimethylamine hydrochloride. It is seen that the rate of continuous amino acid oxidation is increased in the presence of one equivalent of secondary amine with regard to catechol and possibly slightly more in the presence of two. Still bigger proportions show no noticeable further increase and possibly a slight decrease if the excess of secondary amine becomes too big. The main difference in the oxygen uptakes occurs

during the initial period of rapid oxidation when the oxidizing system is being built up. Since the oxygen uptake of catechol alone is not increased by any excess of secondary amine it may be said that in the presence of the latter the oxidation of amino acid starts earlier and that a bigger or more efficient amount of the oxidizing system is formed.

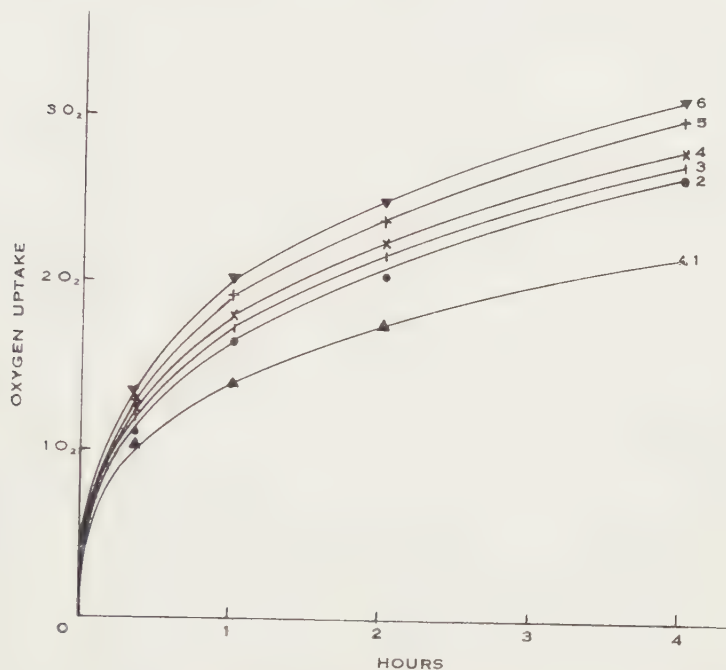


Fig. 9.—Effect of varying amounts of secondary amine on the oxidation of glycine. Warburg, 37°C.; M/15 phosphate buffer pH 7.8; 100 shaking. All flasks contained 1 ml. M/100 catechol and 1 ml. M/10 glycine. The quantities of dimethylamine hydrochloride were:

Curve 1: Nil.

Curve 2: 0.1 ml. M/10 dimethylamine hydrochloride.

Curve 3: 0.2 ml. M/10 dimethylamine hydrochloride.

Curve 4: 0.4 ml. M/10 dimethylamine hydrochloride.

Curve 5: 2.0 ml. M/10 dimethylamine hydrochloride.

Curve 6: 8.0 ml. M/10 dimethylamine hydrochloride.

IV. CONCLUSIONS

(a) Colour Compounds

(i) *Formation of the Mono-Substituted Colour Compounds.*—No evidence could be found that 1,2,4-trihydroxybenzene is a necessary intermediate in the formation of the colour compounds. The observations recorded are accepted as showing that the mono-substituted compounds are formed by combination of *o*-quinone with the amine or amino acid.

The reaction between quinones and aniline can proceed in two different courses (cf. Karrer 1938, p. 532): (1) *Addition* of the amino group to a double bond, followed by rearrangement of the molecule to the dihydroxyphenol and *re-oxidation at the phenolic groups with uptake of one atom of oxygen*. The original *ortho*- or *para*-quinonoid structure is thus preserved; the formulae of

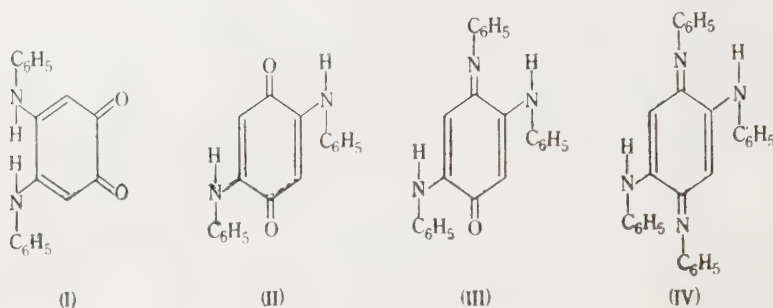


Fig. 10

4,5-dianilino-*o*-quinone (I) (Kehrmann and Cordone 1913) and of 2,5-dianilino-*p*-quinone (II) (Hofmann 1863) are given in Figure 10. (2) In the presence of excess aniline, *condensation with a keto group* of the quinone takes place, dianilino-*o*-quinonemonanil (III) (Fincke and Hagen 1885) and dianilino-*p*-quinonedianil (IV) (Kimich 1875) being formed. *No oxygen is consumed* in this reaction. All these compounds are intensely coloured. Compounds analogous to the aniline compounds have been prepared from *p*-quinone and dimethylamine (Mylius 1885) and other secondary amines; it is suggested that the corresponding compounds from *o*-quinones are the colour compounds in question.

Of the two atoms of oxygen consumed by catechol in the formation of the mono-substituted colour compounds, one is accounted for by the oxidation of catechol to *o*-quinone. The second atom of oxygen is consumed in the combination with the amine, which is therefore considered as an *addition* to one of the double bonds to form first the *leuco compound* which is then further oxidized to the *oxy compound*. It is supposed that the *ortho*-quinonoid structure is preserved exactly as it is in the reaction products of *o*-quinone with aniline. Figure 11 shows the oxidation of catechol alone (Baldwin 1947) and that of dopa and the formation of the colour compound from catechol and glycine presented as parallel reactions with identical oxygen uptakes.

No evidence was found that, in the reaction with non-oxidizable amines or amino acids, more oxygen is consumed than one atom of oxygen for one molecule of catechol. The additive reaction stops with the combination of *o*-quinone with one molecule of amino compound; the reactivity of the second double bond appears to be weakened through the first substitution.

(ii) *The Di-Substituted Colour Compounds*.—The curves of the colour intensity obtained by the oxidation of catechol with varying proportions of

secondary amine or secondary amino acid show a distinct maximum at the proportions of two of amine to one of catechol. The observation is taken as suggesting that a further molecule of the amino compound entered into the reaction. Since no further oxygen uptake occurred, it is supposed that this

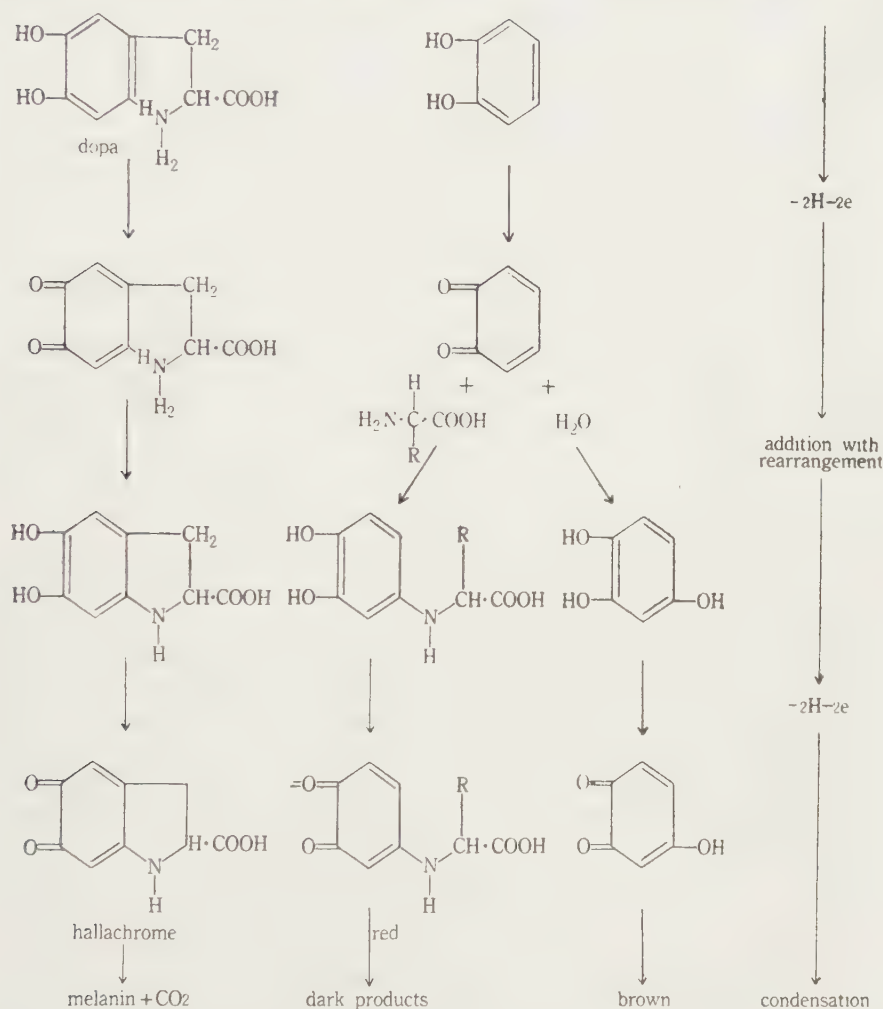


Fig. 11.—Steps of the enzymic oxidation of dopa, of equimolecular proportions of catechol and glycine, and of catechol alone.

combination is a *condensation* between one of the keto groups of the *o*-quinone and the secondary amino group, analogous to the formation of anils in the reaction between quinones and aniline. No conclusive evidence could as yet be obtained to suggest which of the keto groups is reacting. Tentatively the 2 position is proposed and accepted for the formulations of the compounds shown in Figure 12; (I) and (II); similar *p*-quinone-immonium structures are present in indamines, toluene blue, etc. Close homologues are dimethylamino-

p-quinone-immonium perchlorate and picrate (Piccard and Larssen 1918, Fig. 12, III). It appears that, *just as only one double bond of the o-quinone is capable of the addition reaction, so only one keto group is capable of the condensation reaction.* (The limitation need not extend to different experimental conditions.)

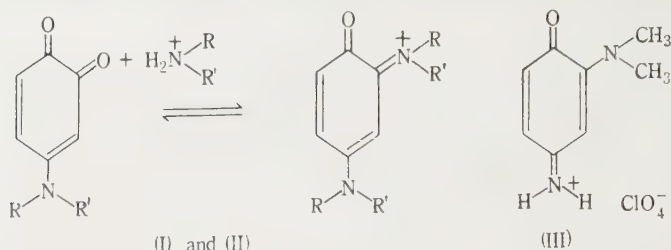


Fig. 12

The curves of colour intensity appear to show a slight kink at equimolecular proportions of catechol and secondary amine, indicating possibly that the reaction proceeds stepwise: first the addition of one molecule of amine to one double bond with subsequent re-oxidation of the molecule, and then the condensation of a further molecule of the amine with a keto group of the mono-substituted compound and without further oxygen uptake.

The smooth curves of colour intensity formed in the oxidation of catechol in the presence of varying amounts of oxidizable or non-oxidizable primary amino acids do not give any indication of the formation of a condensation compound. Its formation, though possibly only to a small extent, is, however, suggested by the observations that, on addition of an oxidizable primary amino acid to the purple di-substituted compound formed with secondary amines the colour changes slowly to a more brownish tint similar to that obtained in the formation of the colour compounds from primary amino acids, and that small amounts of volatile secondary amines are released. It is supposed that the primary amino acid displaces some of the secondary amine from the di-substituted colour compound and enters the molecule at the point of displacement. Since the second molecule of a volatile secondary amine is less firmly held than the first, it is accepted that the displacement of the volatile amine by the non-volatile oxidizable amino acid takes place at the point of condensation.

It is suggested that, in the presence of sufficient amino compounds, di-substituted colour compounds are formed from amino acids as well as from secondary amines; that all these di-substituted compounds exist in equilibrium with their components and that with secondary amines the equilibrium is in favour of the di-substituted compound. The smooth curves of the intensity of the colours formed with primary amino acids are considered to be due to the facts that the equilibrium is less in favour of the di-substituted compounds and that the compounds are less stable than those from secondary amines. It is held that the formation of a di-substituted compound constitutes the first step towards the oxidation of a primary amino acid.

(b) *The Mechanism of Amino Acid Oxidation*

It is suggested that the first step of amino acid oxidation consists in a rearrangement of the condensation compound from the quinone-imine to the imino acid structure (Fig. 13), analogous to the mechanism suggested for transamination by glutamic or aspartic transaminase (Snell 1945; Schlenk and Fischer 1947). It is further suggested that the quinone-imine is then hydro-

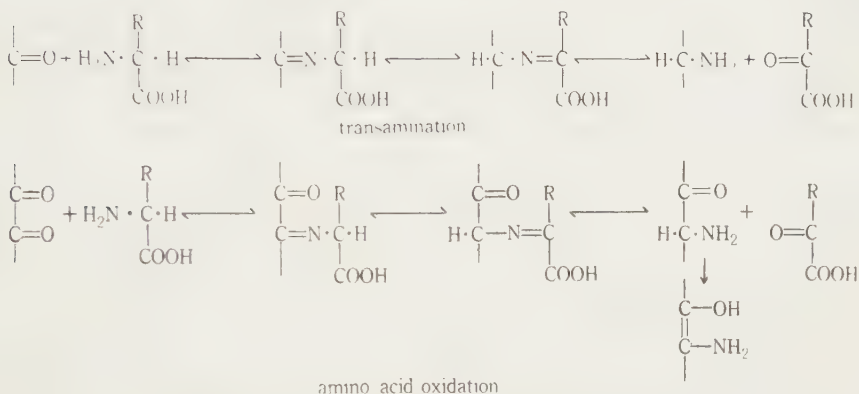


Fig. 13.—Transamination. Amino acid oxidation.

lysed into keto acid and amino phenol and the amino phenol oxidized with liberation of ammonia, thus re-forming the original mono-substituted *ortho*-quinone. The oxygen uptake for this reaction sequence, one atom of oxygen per molecule of the reduced compound, accounts for the oxygen uptake measured in the manometric experiment per molecular equivalent of oxidized amino acid. The continuous oxidative deamination of amino acids is tentatively represented by the cycle shown in Figure 14, taking catechol and glycine as examples. The same formulation can be applied to the oxidation of amino acids by adrenochrome or hallachrome, by Szent-Györgyi's tyrins, by vitamin K (Schönberg, Moubasker, and Said 1949), or other polyketo compounds, a survey of which is given by Lynen (1941).

(c) *Comments on some Single Steps of the Cycle*

(i) *Nitrogen Substitution in the 4 Position.*—The nitrogen in the 4 position does not seem to play an active role in the subsequent reaction but to serve only in the stabilization of the *o*-quinonoid structure and in the preservation of its reactivity through repeated reductions and re-oxidations. It is considered that this effect is due less to the nature than to the fact of a substitution in the vulnerable *para* position and that carbon substitution in the same position of the naturally occurring catechins or of protocatechuic, caffeic, quinic acids etc., has the same effect. With the catechol compounds there is one further point: catechol itself is, at physiological pH in the absence of a catalyst, practically not oxidized by atmospheric oxygen, while the leuco forms of the mono-substituted colour compounds are readily re-oxidized by air. This allows a

rapid reaction in the non-enzymic oxidation and an easy investigation of the system; it may have some physiological significance. The substituted compounds may be more stable and at the same time more efficient.

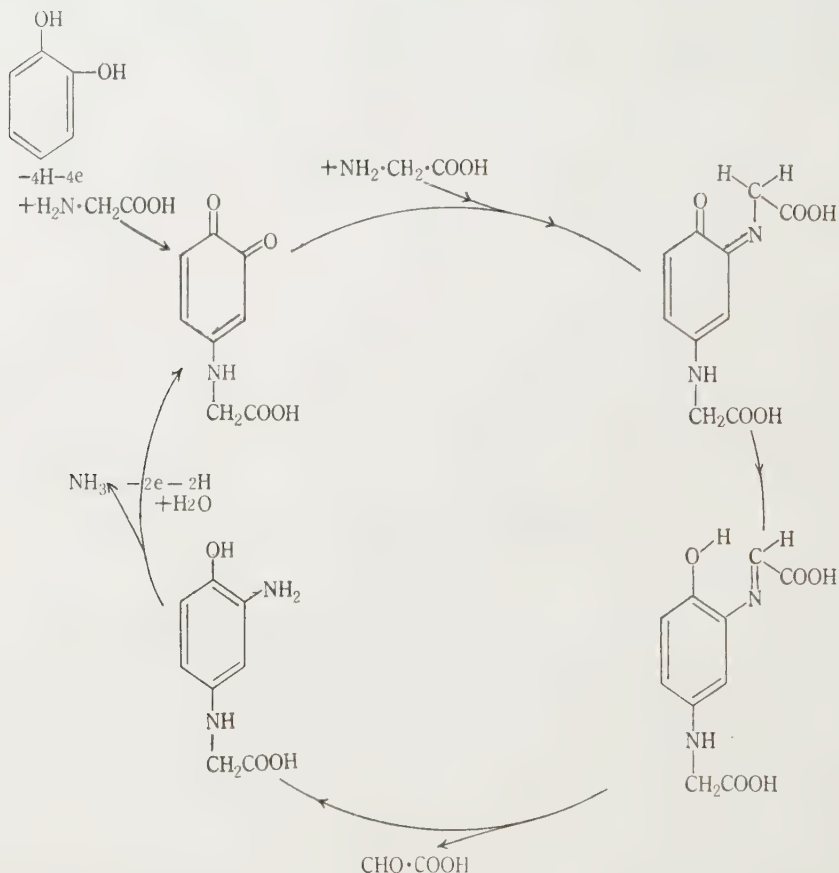


Fig. 14.—Tentative scheme of the continuous oxidation of amino acid by the mono-substituted o-quinonoid colour compounds.

(ii) *The Formation of the Condensation Compound.*—Neither in the slow oxidation of amino acids by the catechol system nor in that by adrenochrome could a difference in colour or any other changes be observed which would indicate the formation of the condensation compound. This step can, however, be supported by observations with another polyketo compound. Alloxan (in acetic acid) oxidizes amino acids with formation of a red intermediate; the lower aldehyde, carbon dioxide, and some ammonia are formed and murexide crystallizes from the reaction mixture (Traube 1911). Small amounts of uramil could be identified, but neither dialuric acid nor alloxanthin could be found. The formation of uramil proves the transfer of ammonia from the amino acid to alloxan and indicates the position at which the interaction takes place (Fig. 15). It is suggested that the red intermediate (which could not be separated

in a pure state) is the condensation compound (see Fig. 15°) corresponding to the di-substituted colour compounds formed with secondary amines and supposed for primary amino acids.

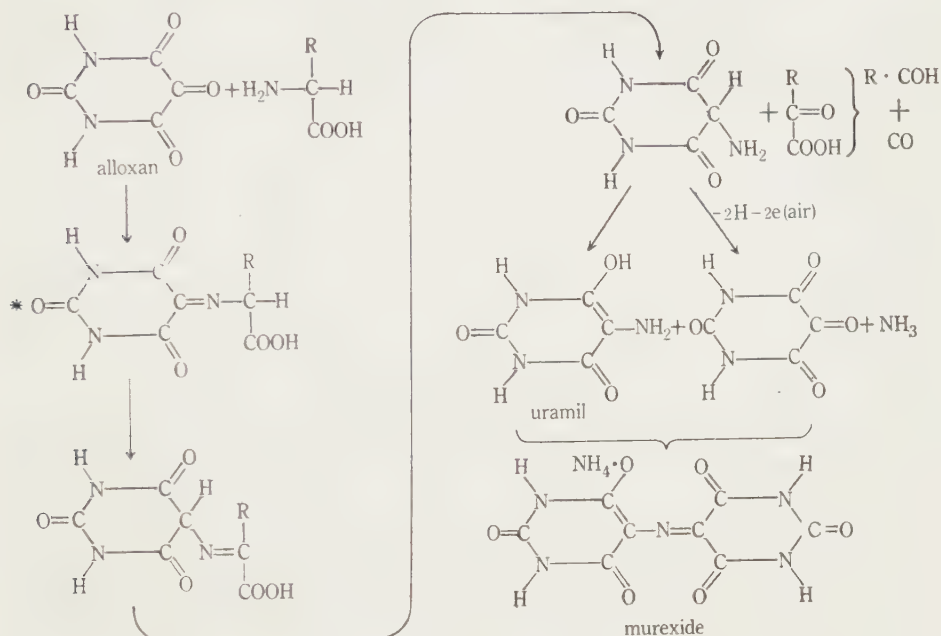


Fig. 15.—Amino acid oxidation by alloxan.

The keto acid decomposes in the boiling mixture to the next lower aldehyde and carbon dioxide; no oxygen is consumed in this step, which is a pure transamination analogous to the first step of the cycle presented. The uramil formed, however, reacts in the boiling mixture with oxygen to form ammonia and alloxan; this reaction can be presented as analogous to the last steps of the cycle. A side reaction occurs in so far as uramil, ammonia, and alloxan combine to form murexide which crystallizes from the boiling mixture and removes the catalyst. Therefore, only "some" ammonia is found in the reaction products and the process is not continuous since the oxidation of each molecule of amino acid removes one molecule of alloxan from the system in the formation of the unreactive murexide. The reaction is of interest because it presents the *formation of the keto acid—or aldehyde plus carbon dioxide—as a distinct and separate step preceding the liberation of ammonia and independent of it.*

(iii) *The Liberation of Ammonia.*—Amino phenols form on mild oxidation quinone-imines which are readily hydrolysed into quinones and ammonia. It may be assumed that in the very dilute solutions of the manometric experiment the conditions for mild oxidation are fulfilled. There are, however, some observations which suggest an alternative course for the reaction. If isatin is

glycine, five to ten times excess of the acid over the catechol present is needed to obtain a brisk rate of amino acid oxidation.

The rearrangement of the condensation compound to the imino acid structure is presented as a proton migration. Dimethylamine and other secondary amines do not possess an active alpha hydrogen atom as do the amino acids. *The oxidation of secondary aliphatic amines cannot, therefore, be expected, even if they form a quinone-immonium structure.*

The presence of an alpha hydrogen is not, however, sufficient to effect the rearrangement of the molecule and the subsequent deamination of the amino acid. James *et al.* (1948) found that their belladonna enzyme oxidized only glycine, ornithine, and a few other amino acids at a noticeable rate; the *Duboisia* enzyme used by the present authors shows the same limitations. Identical observations were made in the non-enzymic oxidation of amino acids by various quinones and related compounds; each system oxidizes some amino acids quickly, some sluggishly, and others not at all. The oxidizable acids are not the same in all cases, but usually the simpler acids are more readily oxidized. The varying reactivity of the alpha hydrogen almost certainly constitutes another factor determining whether a given amino acid is oxidized at an appreciable rate by the system.

The rate of continuous oxidation of a given amino acid under otherwise identical conditions appears thus to depend on the extent to which the mono-substituted compound is formed and capable of preserving its reactivity through various reductions and re-oxidations, on the extent to which the quinone-imine structure is formed, and on the reactivity of the alpha hydrogen of the amino acid.

V. DISCUSSION

It must be emphasized that the argument presented for the chemical mechanism of continuous amino acid oxidation by the catechol-oxidase system applies only to concentrations and conditions similar to those of the enzymic reaction. Quinones are strong oxidizing agents. Dry *o*-quinone may decompose with explosive violence in contact with the same compounds (e.g. phenylhydrazine) which in dilute solution reduce it smoothly to catechol. The observations presented on the stability of the compounds, the limitation of quinone-imine formation and the necessity of an alpha hydrogen do not apply to more concentrated solutions, to reaction at higher temperature, or to extremely slow reactions. At more alkaline pH, above 10, the colour compounds formed from catechol and amino acids as well as adrenochrome undergo further oxidation; the colour compounds from some amino acids, e.g. proline, which are not noticeably oxidized under aerobic conditions lose their colour if the access of air is restricted and regenerate it on admission of oxygen, suggesting a slow oxidation of the amino acid; α -aminoisobutyric acid, which has no alpha hydrogen, is oxidized within eight days by *p*-quinone to acetone, carbon dioxide, and ammonia (Langenbeck 1928); and benzylamine, which has neither an alpha hydrogen nor a carboxyl group, is oxidized by isatin or alloxan

to ammonia and benzaldehyde (Traube 1911). The reaction studied in this paper takes place at room temperature and the result was considered to be negative if within 4-5 hours no noticeable oxidation was observed.

No observations were made to suggest that the colour compounds are formed through the intermediate 1,2,4-trihydroxybenzene; the evidence points to the assumption that at neutral and slightly alkaline pH in the presence of secondary amine or any amino acid the colour compound is preferentially formed and not trihydroxybenzene or any other catechol oxidation product. These colour compounds are more stable than the catechol oxidation products which rapidly lose their quinonoid reactivity.

It appears that the mechanism of enzymic oxidation is more complex than the formulation by Nelson and Dawson (1944) tends to suggest. Catechol is first oxidized to *o*-quinone and this consumes one more atom of oxygen, but the mechanism of this second step could not be duplicated by starting with the same concentrations of 1,2,4-trihydroxybenzene. Furthermore, the quinonoid reactivity is somehow lost during the second step of catechol oxidation. Willstätter and Müller (1911) succeeded in isolating the dimers of more stable substituted (3- and 4-methyl- and 4-methoxy-)*o*-quinones as yellow crystals and found that these no longer showed the quinone reactions with guaiacum and hydriodic acid given strongly by the monomers. It is tentatively suggested that *the oxidation products of catechol beyond the o-quinone stage present the oxidation levels of trihydroxybenzene and finally hydroxyquinone, but no longer the monomolecular structure* and that this di- or polymerization is responsible for the early loss of quinonoid reactivity, of the capability of forming the colour compounds, and of oxidizing additional amino acid.

It is suggested that, in the presence of amino acids or secondary amines, the *o*-quinone formed in the oxidation of catechol adds first one molecule of the amino compound to one of the double bonds; through rearrangement and oxidation the nitrogen substituted *o*-quinonoid colour compound is formed. All these colour compounds are presented as structural homologues; the substance tyrin described by Szent-Györgyi (1925) appears to cover individual members of this group formed from amino acids and such substituted catechols, catechins, their glucosides, or related compounds as are present in plant and possibly animal tissue.

The mechanism of continuous amino acid oxidation is presented as a condensation of the amino group of a primary amino acid with one of the keto groups of the quinone, followed by rearrangement, hydrolysis, and re-oxidation. With *o*-quinone this reaction is preceded by the addition of one molecule of amino compound to one of the double bonds followed by oxidation to a substituted *o*-quinone. No role is ascribed to this substitution in the 4 position other than that of stabilizing the compound. Alloxan, isatin, and ninhydrin do not possess double bonds which are reactive under the conditions of the experiment; therefore they do not form an addition compound, but react directly by condensation with one of the keto groups, oxidizing the first molecule of amino acid with which they combine.

In the reactions discussed the two keto groups are in the *ortho* position to each other. This is not a necessary condition. *p*-Quinone oxidizes certain amino acids, though considerably more slowly than the system described here (Langenbeck 1928). The question whether the reaction proceeds depends probably not so much on the position of the two keto groups as on their reactivity.

The discussion of the extent of the physiological function of the reaction is outside the scope of this investigation except for the following consideration: it is suggested that, in the presence of amino acids or secondary amines, *o*-quinones with free *para* positions react to form the coloured compounds described; but it is not suggested that the nitrogen substitution is necessary for the stability of the redox system, nor that catechol or its simpler homologues are the natural substrates of the system. Boswell and Whiting (1938) separated from potato tubers an unidentified catechin whose action with potato tyrosinase was identical to that of catechol or protocatechuic acid; the present authors extracted from belladonna root a catechol compound, probably a catechin, which could replace catechol in the enzymic reaction and was even superior in its action; the amounts isolated were too small to allow further identification. It can be accepted that these compounds, although forming redox systems of equal or similarly high potential, are smoother in their action and more easily controlled than the violently reactive simple *o*-quinones.

VI. ACKNOWLEDGMENTS

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RUMINAL FLORA STUDIES IN THE SHEEP

II. THE EFFECT OF THE LEVEL OF NITROGEN INTAKE UPON THE TOTAL NUMBER OF FREE MICROORGANISMS IN THE RUMEN

By R. J. MOIR* and V. J. WILLIAMS*

[*Manuscript received March 6, 1950*]

Summary

A method of obtaining ruminal samples for the investigation of the "free" microorganisms of the sheep's rumen by means of a stomach-tube is described.

The total numbers of microorganisms per unit volume obtained in this way were found to be lower than in samples taken directly from whole-rumen contents. The correlation between the counts for the two series of samples and the constancy of the difference is considered to justify the stomach-tube method as a means of assessing changes in the number of microorganisms in the rumen.

The data are presented from a feeding trial designed to determine the effects of varying intakes of protein upon the number of ruminal microorganisms. In this trial five sheep were fed five levels of protein derived from casein and oaten hay. The levels of protein consumed were approximately 3, 6, 9, 10, and 12 per cent. of the dry matter of the ration.

An extremely high correlation ($r = +0.98$) was found between the levels of intake of protein and the numbers of microorganisms in the rumen. It was concluded that, under the conditions of the experiment, the number of organisms was determined by the protein intake.

The regressions for the numbers of organisms against protein nitrogen are presented. These indicate that a relatively constant proportion of the ingested protein (of the order of 50 per cent.) was converted to bacterial protein.

These findings, together with the "true" digestibility and biological value of the protein of each ration, are discussed in relation to protein metabolism in the ruminant.

I. INTRODUCTION

During the last decade the functional importance of the indigenous microflora and microfauna of the rumen has been fully recognized and considerable attention directed to elucidating their precise roles in ruminant metabolism. The spectacular success achieved by these animals in utilizing insoluble polysaccharides as a source of energy has naturally attracted many investigators, and notable advances have been made in our understanding of the parts played by the ruminal microorganisms in this process. The position with respect to the utilization of the nitrogenous constituents of the feed is much less satisfactory, although it is obvious that the whole microbial population must participate in some way in protein metabolism since ingested protein is normally its major source of nitrogen.

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The fact that ruminal microorganisms must obtain all or most of their nitrogen from the food suggests that, in the presence of adequate available energy and other nutrients, the intake of nitrogen will determine the extent of the proliferation of these organisms. Little direct experimental evidence upon this point was available before the experiments described in this paper. Smith and Baker (1944) showed that, where ammonium salts or urea were fed as the main source of nitrogen, multiplication was limited by energy supplies. van der Wath (1942) found that the addition of dextrinized starch to a basal ration of poor wheat straw did not raise the ruminal bacterial count, whereas the further addition of urea raised it by 74 per cent. Replacement of the urea by fishmeal increased the count to three times the original figure. van der Wath and Myburgh (1941) demonstrated a considerable seasonal fluctuation in the numbers of ruminal bacteria in grazing sheep in South Africa. They suggested that the protein content of the pasture influenced the numbers considerably. This suggestion was later supported by the work of Louw and van der Wath (1943), who added meatmeal to a ration of poor prairie hay containing 3 per cent. protein and obtained a highly significant increase in the numbers of ruminal bacteria. Replacement of varying proportions of meatmeal with maize resulted in minor increases in the counts until the maize supplement reached 150 g. Further additions of maize in the absence of meatmeal, and particularly when 300 g. were fed, caused a decline in bacterial numbers.

These findings indicate that the level of protein ingested can affect the numbers of microorganisms within the rumen. The results of an experiment designed to reveal whether any direct quantitative relationship exists between protein intake and numbers of ruminal microorganisms in the sheep are presented in this paper.

II. RUMINAL SAMPLING TECHNIQUE

(a) *The Sampling Method*

The use of a stomach-tube as a means of obtaining ruminal samples, rather than fistulated animals, was investigated. The path taken by tubes passed into the oesophagus was described by Watson and Jarrett (1945), who showed that the tube, unless accompanied by a liquid, passes into the rumen or reticulum.

Tubes of 6 to 7 mm. diameter were introduced through the nostril and were found to pass readily into the oesophagus. Much larger tubing can be passed by way of the mouth. The minimum diameter that could be used without blocking, particularly in grazing animals, was 6 to 7 mm. After carefully introducing the tube 27 to 30 in., a suction pump was attached and negative pressure applied. A trap in the line was employed so that the ruminal sample was withdrawn directly into a large sample bottle. A quantity of this material was immediately diluted with half its volume of 50 per cent. formalin. In this state, samples remained unchanged for a considerable period.

While it was recognized that this method of sampling is not ideal, it was considered that the samples taken should reflect any changes taking place in the total numbers of ruminal microorganisms. Baker (1943) showed that, in the ox, the proportion of solid to liquid fraction diminishes progressively with the depth. Further, the efficiency of the stomach-tube in gathering representative samples was strongly doubted by Gall, Stark, and Loosli (1947), who found that the count on ruminal material obtained in this manner was $27 \pm 2.23 \times 10^9$ per cc. compared with $93 \pm 19.5 \times 10^9$ per g. for solids and $62 \pm 9.3 \times 10^9$ per cc. for liquid withdrawn from a fistula.

In view of these results, a series of samples was taken by intubation from a number of sheep which were then immediately killed and further representative whole-rumen samples obtained. Counts were made on these for total numbers of microorganisms, the results of which are given in Table 1.

TABLE I
COMPARISON OF TOTAL COUNT OF MICROORGANISMS IN WHOLE-RUMEN AND STOMACH-TUBE SAMPLES

Sheep No.	727	728	729	730	731	732	X	A5	Mean
Whole-rumen sample	42.8	51.1	61.5	63.5	44.1	45.2	66.5	83.5	59.3
Tube sample	31.5	57.6	53.4	47.7	38.8	29.6	53.9	66.1	49.3

Correlation coefficient of whole-rumen sample on tube sample = 0.84 with 99 per cent. probability.

Per cent. difference between whole-rumen sample and tube sample -17.25 per cent. significantly constant at 99 per cent. probability.

The constancy of the difference and the highly significant correlation between the two series of samples show that the stomach-tube sample reflects accurately the state of the free microorganisms within the rumen, and justifies this method of sampling where comparisons of total counts are being made.

To check the uniformity of samples taken by intubation, series of three samples were taken from five sheep, the tube being withdrawn and reinserted for each sample. In four of the five sheep a high degree of uniformity was obtained. In subsequent work, the agreement between samples from the same sheep under the same dietary conditions, even when taken several days apart (usually on the 7th and 10th day of trial) has been found to be excellent.

(b) Time of Sampling

Johnson *et al.* (1944) made plate counts in ruminal samples at hourly intervals after feeding. The results obtained by this method showed the bacterial counts to be highest one hour after feeding. Thereafter they rapidly declined. The production of methane followed a similar pattern and was taken to corroborate the evidence of the plate counts.

Direct counts on a series of samples taken at four-hourly intervals from a pen-fed sheep in this laboratory indicated that the maximum total number

was reached between four and eight hours after feeding and was maintained at this level for some hours. The work of Hale, Duncan, and Huffman (1947) on ruminal digestion indicated that the "digestion" of protein and soluble carbohydrates reached a maximum within six hours of feeding, and that the "digestion" of cellulose was approaching its maximum at that time. Very little change was found after 12 to 14 hours. It was considered on the basis of this evidence that the total numbers would be close to a maximum at six hours. All samples were consequently taken six hours after feeding and, with very few exceptions, excellent agreement has been found between samples taken on different days at this time.

III. EXPERIMENTAL PROCEDURE

(a) *Design of Experiment*

The feeding trial was designed on the basis of a 5×5 latin square in which five sheep, five trials, and five rations of increasing protein intake were used. This layout was adhered to as far as the feeding of the rations was concerned, but it was found that feed refusals so modified the intake of protein that it became necessary to regroup the results according to the actual nitrogen intake.

(b) *Experimental Animals*

Five two-year-old Romney Marsh x Merino comeback wethers of similar breeding were selected from available animals on the basis of weight and temperament.

(c) *Rations*

The five rations were compounded so that they would be of equal dry matter and crude fibre content, and of similar gross energy value. The main variable in the rations as fed was the crude protein content, which was increased from 3.7 to 18 per cent. in 3.5 per cent. steps. The lowest protein ration was made up of 500 g. of chaffed oaten hay and 173 g. of starch, a total of 673 g. of dry matter fed daily. The protein of the other rations was increased by replacing the starch with an equal weight of casein. The casein in these rations supplied from one-half to four-fifths of the total protein intake. The composition of the diets and their crude protein content are given in Table 2.

All the rations were mixed and weighed into individual paper bags before the commencement of the trial to ensure minimum variation. The rations, together with 6 g. each of sodium chloride and calcium carbonate, were fed daily at 9 a.m.

The chaff of the basal ration had been poorly cut, permitting some selection and scattering and led to some small errors in collecting feed refusals. Some feed refusals were experienced on all rations, causing an overall reduction in protein intake. The actual levels of protein intake became 3, 6.2, 8.7, 9.5, and 12 per cent.

(d) Trial Regime

Two weeks before the trial all sheep were placed on the basal ration, and the five selected sheep then given the trial rations. In each trial the preliminary feeding period was 14 days, followed by a 10-day collection period in metabolism crates. Where sheep have been continuously on dry feed for several months, 14 days was found to be sufficient to allow adjustment of the balance of the ruminal microorganisms. If, on the other hand, sheep grazing on green material are brought in, a period of at least six weeks is probably required.

TABLE 2
COMPOSITION OF DIETS - DRY BASIS

Diet	Dry Matter (g.)	Oaten Hay (g.)	Starch (g.)	Casein (g.)	Nitrogen (%)	Crude Protein (%)
A	672	499	173	0	0.595	3.72
B	672	499	149	24	1.157	7.23
C	673	499	124	50	1.734	10.84
D	673	499	99	75	2.294	14.34
E	674	499	73	102	2.893	18.08

(e) Collections

Collections of the urine, faeces, and food residues were made daily at 9 a.m. An aliquot of these materials was taken, bulked, and stored until the end of the trial. The faeces and residue samples were oven-dried daily. Ruminal samples were taken on the fourth, seventh, and ninth days of the collection period and treated as previously described. Counts were made on the two later samples only. The values presented are the mean of these two counts.

(f) Counting Technique

A Petroff-Hauser bacterial counting chamber, as described by van der Wath (1948), was used in making the counts. After thoroughly shaking the fixed ruminal sample, a final dilution of 1 in 500 was made, using a Thoma pipette and a 0.1 per cent. solution of aniline blue. After thoroughly mixing, the chamber of the counting cell was filled and placed immediately on the stage of the microscope. The preparation was examined, and any showing wide variation or incomplete dispersion was discarded. A dry combination of lenses, 15x oculars and one 60x achromatic objective, was used for all counts, and the number of organisms in 80 small squares counted. The total number always exceeded 200. From the number of organisms counted, the volume of the chamber, and the dilution, the total number per cubic millimetre was calculated.

IV. RESULTS

The data gathered from the trials are presented in Table 3 in the order in which the rations were fed. As can be seen from the results, feed refusals considerably modified the intended protein intakes, and consequently the data

were regrouped on the basis of actual nitrogen intakes. These groups are given in Table 4, together with the nitrogen intake and balance, "true" digestibility of the nitrogen, biological value, and the total number of organisms as determined in the counts.

TABLE 3
NITROGEN BALANCE RESULTS PER 10-DAY PERIOD. BACTERIA PER CU. MM.

Feed	Sheep	Average Body Wt. (lb.)	Dry Matter Intake (g.)	Nitrogen Intake (g.)	Faeces Nitrogen (g.)	"True" Digestibility of Nitrogen ^a (%)	Urine Nitrogen (g.)	Per cent. Urine Nitrogen of Nitrogen Intake	Nitrogen Balance (g.)	Biological Value ^a (%)	Microorganisms in Rumen (millions per cu. mm.)
Trial 1											
A	732	88	5770	29.0	43.13	62	14.82	51	- 28.95	80	31
B	730	83	6410	61.0	41.25	91	29.76	49	- 10.01	62.5	41.5
C	731	78	6360	98.0	42.46	93	44.77	46	+ 10.77	62	53
D	728	91	6740	128.0	48.5	91.5	81.7	64	- 2.2	40	61
E	729	87	6350	122.0	44.94	92	74.35	61	+ 3.71	43	61
Trial 2											
A	731	70	5040	24.5	33.96	76	12.0	49	- 21.42	84	28.5
B	728	91	6710	67.2	49.34	96	30.96	46	- 13.1	64	40
C	729	86	6730	101.0	43.31	91	43.45	43	+ 14.25	66	57
D	732	88	6215	87.4	36.9	96	44.29	51	+ 6.21	63	57
E	730	86	6190	112.0	42.47	93	73.87	66	- 4.44	39	55
Trial 3											
A	728	85	4400	15.3	27.25	82					25
B	729	91	6730	67.4	42.0	93	34.66	51	- 9.26	63	42
C	730	88	6730	101.0	42.82	94	43.2	43	+ 14.98	66	56
D	731	74	6180	122.9	45.42	91	80.13	65	- 4.20	37	62
E	732	87	4750	77.9	31.88	93	39.26	50	+ 6.8	60	55
Trial 4											
A	730	86	4780	27.8	33.81	74	14.5	52	- 20.57	82	26
B	731	77	5090	50.9	31.87	93					34
C	732	91	5675	73.6	37.44	92	37.53	51	- 1.4	61	56
D	729	95	6660	119.0	47.77	91	75.5	63	- 4.4	41	62
E	728	90	6120	80.1	40.44	92	38.54	48	- 1.12	63	51
Trial 5											
A	729	91	4740	23.9	33.5	70	13.39	55	- 22.9	86	35
B	732	93	5895	60.8	37.1	93	31.4	52	- 7.7	65	42
C	728	94	6505	89.9	43.4	92	45.0	50	+ 1.5	60	55
D	730	87	6720	125.9	49.0	91	75.96	60	+ 1.06	43	62.5
E	731	78	6150	133.0	48.3	89	85.4	64	- 0.7	35	64

^a Assuming metabolic nitrogen of faeces = 5.563 mg. per g. dry matter intake and endogenous nitrogen of urine = 1.252 g. per 100 lb. live weight (Hamilton, Robinson, and Johnson 1948).

Figures 1 and 2 show graphically the peculiarity of the behaviour of Group 5.

(a) Nitrogen Balances

Group 5 was found to have a significantly lower nitrogen balance than Group 4 ($P < 0.01$) but was not different from Group 3. There is a very high correlation between the nitrogen intake and the nitrogen balance for Groups 1 to 4, the regression for the relationship (excluding Group 5) is nitrogen balance = $-35.75 + 0.45 x$, $r = 0.98$.

TABLE 4
MAIN EXPERIMENTAL RESULTS

Sheep	Nitrogen Intake (g.)	"True" Digestibility of Nitrogen (%)	Per cent. Urine Nitrogen of Nitrogen Intake	Nitrogen Balance (g.)	Biological Value (%)	Microorganisms in Rumen (millions per cu. mm.)
Group 1						
732	29.0	62	51	- 28.95	80	31
730	27.8	74	52	- 20.57	82	26
729	23.9	70	55	- 22.99	86	35
731	24.5	76	49	- 21.42	84	28.5
728	15.3	82				25
Group 2						
730	61.0	91	49	- 10.01	62.5	41.5
728	67.2	96	46	- 13.1	64	40
729	67.4	93	51	- 9.26	63	42
732	60.8	93	52	- 7.7	65	42
731	50.9	93				34
Group 3						
732	87.4	96	51	+ 6.2	63	57
728	89.9	92	50	+ 1.5	60	55
728	80.1	92	48	- 1.12	63	51
732	77.9	93	50	+ 6.8	60	55
732	73.6	92				53
Group 4						
731	98.0	93	46	+ 10.77	62	53
729	101.0	91	43	+ 14.24	66	57
730	101.0	94	43	+ 14.98	60	56
Group 5						
731	133.0	89	64	- 0.7	35	64
731	122.9	91	65	- 4.28	37	62
728	128.0	91.5	64	- 2.2	40	61
730	112.0	93	66	- 4.44	39	55
730	125.9	91	60	+ 1.06	43	62.5
729	122.0	92	61	+ 3.71	43	61
729	119.0	91	63	- 4.4	41	62

Figure 2 shows the excessive urinary nitrogen excretion of Group 5. This is significantly different ($P < 0.01$) from Groups 1 to 4 inclusive, and is directly related to the low nitrogen balance and the biological value of the protein in this particular group.

(b) Biological Values

Biological values were calculated assuming the metabolic faecal nitrogen excretion to be 5.563 mg. of nitrogen per g. of dry matter intake and the endogenous nitrogen of the urine to be 1.252 g. per 100 lb. live weight (Hamilton, Robinson, and Johnson 1948).

The calculated biological values of the protein fed at the five different levels fall into three distinct groups, Group 1 (83), Groups 2, 3, and 4 (63.6, 61.5, and 62.6), and Group 5 (39.7). The high values obtained from Group 1 are due to the low intake and the low digestibility of the nitrogen fed. In Group 5 the low values result from the excessive urinary excretion and are no doubt due to inadequate supplies of energy readily available to the ruminal microorganisms, to the host animal itself, or to both. The low nitrogen balance of Group 5, mentioned earlier, is probably due to the same cause (see Figs. 1 and 2).

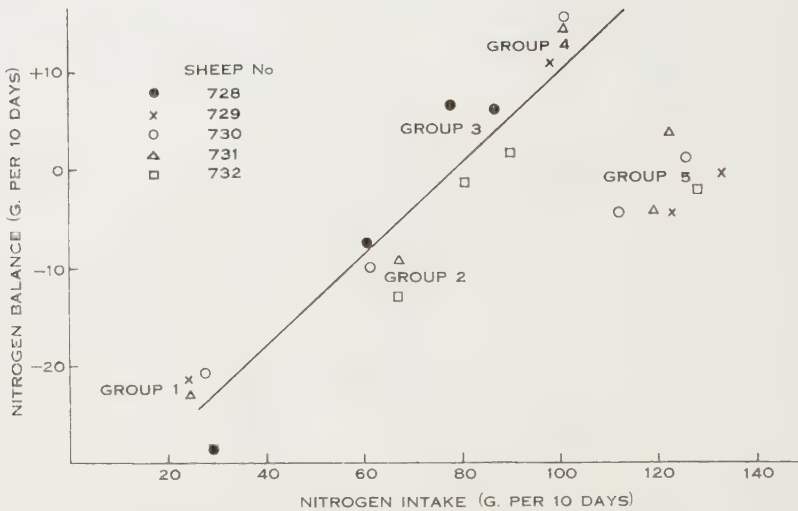


Fig. 1.—Nitrogen balance relationships of the five groups.

(c) "True" Digestibility of Nitrogen

The "true" digestibility of the nitrogen in Group 1, where the sole source of protein was from oat hay, is significantly lower than in the other four groups. The average is 70.5 per cent., the range being 62 to 76 per cent. for the four results. The "true" digestibility of the nitrogen in Groups 2 to 5 is practically constant, the overall average being 92 per cent. with a range of 89 to 96 per cent. for the 18 results. In all these rations the oat hay was supplemented with casein and the very marked effect of this supplementation, particularly in Group 2, is noteworthy.

(d) Counts for Ruminal Microorganisms

The relationship between the bacterial counts and the nitrogen intakes is shown in Figure 3.

Table 5 gives the individual and average regression equations and correlation coefficients for the data. There is no significant difference between the slopes of the individual regressions. This shows that the sheep all responded in the same manner, and the high average correlation coefficient of 0.98 indicates that most of the increase in numbers can be attributed directly to the nitrogen intake.

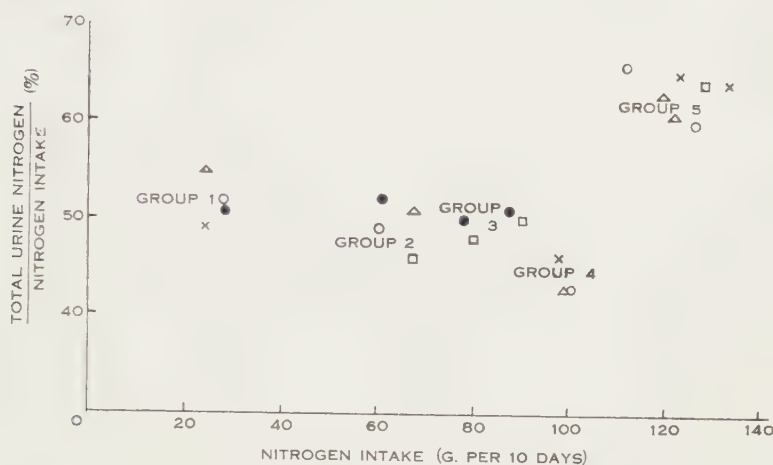


Fig. 2.—Excessive urinary nitrogen excretion of sheep in Group 5.

The variation between individuals is very slight, which is in keeping with the findings of Gall, Stark, and Loosli (1947), and Gall *et al.* (1949) for both sheep and cattle.

TABLE 5
REGRESSION EQUATIONS FOR CORRELATION OF NITROGEN INTAKE ON
MICROORGANISMS PER CU. MM.

Sheep	Equation				Correlation Coefficient	Sum of Squares	D.F.
	D.F.	Y	=	a + bX			
728	4	Y	=	20.82 + 3.36 X	0.97	46.241	3
729	4	Y	=	26.3 + 2.9 X	0.98	20.806	3
730	4	Y	=	17.6 + 3.58 X	0.99	19.217	3
731	4	Y	=	18.44 + 3.47 X	0.996	7.912	3
732	4	Y	=	15.3 + 4.76 X	0.98	17.086	3
Average equation	20	Y	=	20.95 + 3.43 X	0.98	111.262	15

Y = Microorganisms per cu. mm.

X = Nitrogen intake as g. per day.

The numbers of organisms per cubic millimetre ranged from 25 millions on a nitrogen intake of 1.53 g. per day to 64 millions on an intake of 13.3 g. per day. These figures represent largely the "free" organisms rather than the total organisms (which would include organisms attached to plant material) and must be regarded as minima, particularly as samples taken by this method

give lower counts than whole-rumen samples. The magnitude of the counts compares favourably with that found by Gall, Stark, and Loosli (1947), and Gall *et al.* (1949), for winter (non-pasture) ration. The numbers of organisms per cubic millimetre found by these workers, and in the present study, are very much higher than those quoted by Louw and van der Wath (1943), which

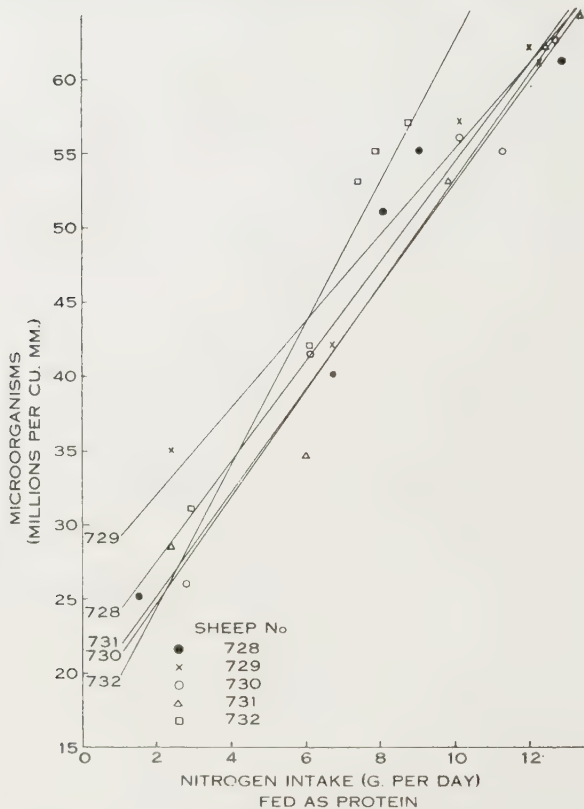


Fig. 3.—Relationship between bacterial count and nitrogen intake for each animal.

range from 1.2 to 1.8 millions (means of groups). This may be due to the lower magnification (Zeiss Huyghen ocular 7x and a Zeiss achromat 40x dry lens) which was used by Louw and van der Wath in their counts.

V. DISCUSSION

The extremely high correlation ($r = +0.98$) found between counts for the total number of free microorganisms in the rumen and the daily intake of nitrogen in the form of protein indicates that, under the conditions of the experiment, the rise in the count from 25 to 64 millions per cu. mm. can be attributed almost entirely to the level of protein ingested. The constant increase in the numbers of the microorganisms with increasing protein intakes, from 3 to 12 per cent. of the dry matter of the rations, suggests that a constant

proportion of the food protein was converted to bacterial protein. The regressions (Fig. 3) show that the fivefold increase in protein intake yielded only a 250 per cent. increase in numbers. This suggests a conversion of food protein to bacterial protein of approximately 50 per cent., which is not greatly dissimilar to the figure (about 40 per cent.) found by McDonald (1948), using an entirely different approach and with a ration in which 82 per cent. of the protein consisted of zein. The conversion was not nearly as complete, therefore, as was inferred by Johnson *et al.* (1942) for protein intakes up to 12 per cent.

It will be noted that the level of protein ingested influenced the numbers of ruminal microorganisms similarly over the whole range of protein intake. In Groups 2, 3, and 4, in which the protein levels were approximately 6, 8.7, and 9.5 per cent. respectively, this is perhaps not unexpected in view of the fact that there were no significant differences in the averages of the groups for the "true" digestibility and biological value of the ingested protein. In each of these three groups the average "true" digestibility was very close to 93 per cent. and the biological value to 63. In Groups 1 and 5, however, there were marked differences which call for further comment.

In Group 1, where the protein intake was only about 3 per cent. and came entirely from the oaten hay, the "true" digestibility of this protein was considerably lower (average 73 per cent.) and the biological value appreciably higher (average 83 per cent.) than in the other groups. These figures are strikingly similar to those given by Smuts and Marais (1942) for poor veld grazing where the protein content was 3 per cent. It is apparent that the starch fed in the present experiment had no appreciable effect on the utilization of the limited quantity of protein available. Nor does it seem likely that the starch had any effect on the numbers of ruminal organisms present. As pointed out in the Introduction, van der Wath (1942) found no increase in the counts when dextrinized starch was added to a basal ration of wheat straw, although further additions of urea or fishmeal raised them considerably. It seems clear, therefore, that in Group 1 the number of microorganisms in the rumen was limited by the amount of available protein and not by any energy considerations.

At the highest level of protein intake (Group 5) the protein was not nearly as efficiently utilized and its biological value fell to an average of only 40. For a casein ration supplying similar amounts of protein Johnson *et al.* (1942) obtained a biological value of approximately 60 but the level of available energy was much higher in their experiments than in these. There seems no doubt that the sheep of Group 5 were in negative energy balance and that additional supplies of energy would have improved the utilization of the protein. On the other hand, the increase in the numbers of ruminal microorganisms from Group 4 to Group 5 was of the same order as the increase from Group 3 to Group 4 and Group 2 to Group 3. It seems very likely, therefore, that the ruminal microorganisms in the sheep of Group 5 were able to satisfy their energy requirements from the available materials and that in

this group, as in the others, the numbers were limited by the protein supply alone. The whole question, however, of the influence of varying intakes of protein and energy on the proliferation of the ruminal organisms is under direct investigation at the present time.

VI. ACKNOWLEDGMENTS

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WOOD STRUCTURE IN RELATION TO GROWTH IN *EUCALYPTUS GIGANTEA* Hook. f.

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Summary

A survey has been made of the structure of the wood of *Eucalyptus gigantea* Hook. f. in relation to growing conditions in the Australian Capital Territory. Using the number of cells cut off by the cambium at various intervals during the growing season as a measure of growth, mathematical expressions have been obtained for growth in young trees at ground level, breast height, and half height; from these, growth rate curves have been derived. The time of year at which growth commences, the relationship of vessel production to commencement of growth, the formation of late wood, and the period of dormancy have all been considered in detail. Variations in fibre dimensions have been investigated in relation to growth and it has been shown that in any one growth ring the shortest fibres are found in the early wood and the longest in the late wood. An intrinsic relationship between growth rate and fibre dimensions has been established, and, at the junction between late and early wood, a sudden change in fibre dimensions occurs corresponding to the discontinuity in growth rates. Anomalous woody tissue formed during severe drought conditions is also described.

I. INTRODUCTION

Eucalyptus gigantea Hook. f. (syn. *E. delegatensis* R. T. Baker) occurs in relatively pure stands in the highlands of south-eastern Australia—in Victoria, south-eastern New South Wales, and the Australian Capital Territory at elevations of 3000-4500 ft., and in Tasmania between 1800 and 3000 ft. The size of the tree varies, but on favourable sites it may attain heights up to 300 ft. and stem diameters breast high up to 5 ft. The tree yields valuable timber which has a wide variety of commercial uses. The structure of the wood is of considerable interest in that it shows definite growth rings, a feature possessed by only a few other eucalypts, and also gives an impression of ring-porosity which, however, is quite unlike the well-known ring-porous timbers such as oak, ash, hickory, etc. Examination of cross sections cut from many specimens obtained from Victoria, New South Wales, and the Australian Capital Territory has revealed that the ring of pores observed macroscopically is confined more or less to the early wood and that, in addition, in many growth rings the first-formed early wood is devoid of pores. This is, of course, a distinctive difference from the typical ring-porous timbers in which there is always a ring of very large pores in the first-formed early wood. Another characteristic of the growth ring of *E. gigantea* is that there is, in most cases, a very distinctive band of late wood where again few, if any, pores occur. These two attributes

* Division of Forest Products, C.S.I.R.O., Melbourne.

are most marked in specimens from the mainland but are ill-defined in specimens grown in Tasmania. Comparative photomicrographs as shown in Plate 1 clearly illustrate this point.

It was considered that some information regarding this rather unusual wood structure might be obtained from the systematic examination of the wood laid down during the growing season. It was intended to determine whether, in this species of eucalypt, there is a definite cessation of growth, and when renewal of growth occurs after the formation of late wood. For the purpose young saplings from the Australian Capital Territory were used; these were obtained through the cooperation of the Commonwealth Forestry and Timber Bureau at regular intervals during a period of two years. A record of the wood laid down during the whole growing season and the relationship between growing conditions and types of tissue formed were obtained. In addition it was planned to investigate the variation, if any, in fibre dimensions with respect to growth rate during any one growing season.

There is some importance in determining growth relationships for this species since Moulds (1947) and Jolly (1948) have both drawn attention to the possibility of its use for exotic plantations and its successful employment for exotic reforestation projects has been predicted by Martin (1948) provided that conditions of frost do not fall below 10°F.

II. MATERIAL

From January 1945 to October 1946, with the exception of May, June, and July 1946, three saplings per month were obtained by random selection from an even-aged (approximately 12 years old) regrowth stand of *E. gigantea* in the highlands of the Australian Capital Territory where the forest is of the sclerophyll woodland eucalypt type, the average rainfall 30 in., and the area subject to severe frosts from March to November. In this locality the mean annual temperature is 58°F., ranging from an average minimum of 30°F. in July to an average maximum of 80°F. in January, and the area is sometimes under snow during the winter months. Each sapling was approximately 12 ft. in height and, from each, cross-sectional discs were cut at ground level, breast height, and half height. These discs were used in all examinations. For comparison, specimens of *E. gigantea* from various localities within its range of occurrence were examined; these specimens were taken from the standard wood collection of the Division of Forest Products, C.S.I.R.O.

III. EXPERIMENTAL

Thin cross sections were cut from each disc, care being taken to include the last-formed woody tissue in each case, and these were used for the routine microscopic examinations. In addition to noting the general character of the developing growth rings an attempt was made to gauge the level of cambial activity for each period by counting the number of cells cut off in radial rows subsequent to the formation of the late wood of the previous growing season. Because the growth ring varies in width within the one tree, sections were

taken from what was considered to be the average width of the growth ring and in many cases average counts from opposite sides of the tree were used.

For the work on fibre dimensions, one tree cut in March 1945 was specifically selected and the three discs taken at ground level, breast height, and half height used. From the discs, blocks extending from the pith to the outside of the stem and approximately $\frac{1}{2}$ in. in width, were prepared. From each block a transverse section, 20 μ in thickness, was cut, stained, and mounted in the usual manner. These sections were used for general observation, fibre diameter measurements, and subsequent positioning of the tangential sections cut from the remainder of the blocks. Growth rings were numbered from the bark inwards to the pith 1-11 at ground level, 1-8 at breast height, and 1-5 at half height.

Serial longitudinal tangential sections 80 μ in thickness, were cut from each block, working from the bark side to the pith. From the cross sections referred to above the width of each growth ring was accurately measured and the position of the longitudinal tangential section precisely determined. These were cut approximately parallel to the growth rings, but as the pith was approached the curvature of the growth rings increased and, therefore, the width of the tangential section was progressively reduced in order to retain parallelism to the growth rings. Separation of each section into its component fibres was effected by treatment with equal parts of glacial acetic acid and 100 vol. hydrogen peroxide at 50°C. for 17 hours followed by a further treatment at 100°C. for 2 hours in the same mixture. The supernatant liquid was decanted and then a small quantity of water was added to the treated section. After gentle agitation the fibres readily separated. A suspension of the separated fibres was in each case transferred to a glass slide and fifty fibres chosen at random were measured. The average fibre length for each pre-selected position in the growth ring was thus obtained.

For the determination of radial fibre diameters throughout the growth ring the radial distances occupied by successive groups of five fibres were measured and averaged.

IV. RESULTS AND DISCUSSION

(a) *Growth in Diameter at Various Levels*

The numbers of cells produced radially at the various levels during the growing season are set out in Table 1 and graphically illustrated in Figure 1. For each cell count a different tree was used, hence individual variations in growth rate are responsible for a marked scatter in the results, but because of the number of trees involved the analysis of the results gives a truer picture of growth than would have been the case had all samples been drawn from the one tree. The final values to which the curves in Figure 1 approach were obtained from the averages of cell counts made on 46 trees, using the 1943-44 and 1944-45 growth rings. The numbers of cells in these two growth rings were approximately the same and agreed with the later values recorded for the 1945-46 growth ring. The more apparent features of the growth curves obtained are summarized in Table 2.

For an approximate picture of diameter growth in the bole, and to represent its periodic nature, an equation was derived of the form:

$$y = a + bx + c \sin x + d \cos x + e \sin 2x + f \cos 2x,$$

where y = the number of cells cut off by the cambium at any particular time during the growing period and $x = \frac{360}{\text{year}}$ times the number of days from January 1 (chosen as an arbitrary reference point, see Table 1), x therefore expresses the phase angle of the annual cycle and is measured in degrees. In fitting the equation to the three sets of data for ground level, breast height, and half height respectively, the last three terms did not significantly improve the fit and they were therefore omitted, giving an equation of the form:

$$y = a + bx + c \sin x.$$

TABLE 1
NUMBERS OF CELLS MATURED FROM CAMBIUM INITIALS AT VARIOUS TIMES
DURING THE YEAR

Days	Days Converted to Degrees	Date	Number of Cells Ground Level	Number of Cells Breast Height	Number of Cells Half Height
16	16	16.i.45	103	126	138
33	33	2.ii.45	114	128	179
68	67	9.iii.45	119	130	178
94	93	4.iv.45	167	200	268
121	119	1.v.45	206	217	272
169	167	18.vi.45	153	199	190
196	193	vii.45	113	122	162
227	224	viii.45	193	220	279
258	254	ix.45	203	214	227
285	281	12.x.45	13	23	19
333	328	29.xi.45	71	55	62
353	348	19.xii.45	96	108	108
18	18	18.i.46	136	148	131
53	52	22.ii.46	118	121	135
81	80	22.iii.46	118	162	163
114	112	24.iv.46	184	235	251
205	202	24.vii.46	225	275	275
234	231	22.viii.46	170	173	216
260	256	17.ix.46	171	3	6
284	280	11.x.46	11	20	22

The fact that the term in $\cos x$ is not significant has the interpretation that the date of maximum growth rate does not differ significantly from January 1, nor the date of minimum growth rate from July 1. The fitted curves of the form $y = a + bx + c \sin x$ are not satisfactory since in two cases they show a decrease about the time of minimum growth rate, which is physically impossible. Adjusted curves were therefore fitted with the restriction that the minimum value of the growth rate should be zero (i.e. $dy/dx = 0$, when $d^2y/dx^2 = 0$). As $dy/dx = b + c\pi/180 \cos x$ and $d^2y/dx^2 = -c\pi^2/180^2 \sin x$, minimum growth occurs when $\sin x = 0$, i.e. $x = 180^\circ$ (corresponding to July 1) and $b = c\pi/180$.

The equations for the fitted curves then become:

$$\text{Ground level } y = 86.4 + 0.503 x + 28.3 \sin x,$$

$$\text{Breast height } y = 99.5 + 0.569 x + 32.6 \sin x,$$

$$\text{Half height } y = 112 + 0.668 x + 38.3 \sin x.$$

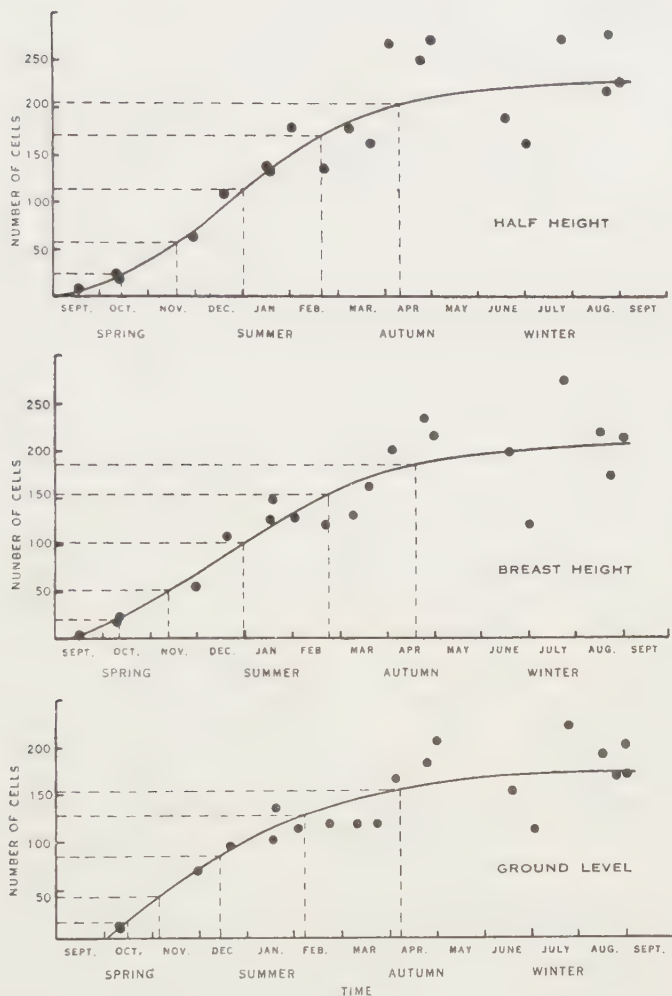


Fig. 1.—Summation curve of growth as determined by cell counts. At half height (upper), breast height (middle), and ground level.

These curves and the derived growth rate curves are shown graphically in Figure 2. From the above equation it is deduced that the new growth ring commences about September 1 and that the period May 1 to September 1, two months on either side of the date of minimum growth rate, may be regarded as a period of little or no growth. From Figure 1 it is apparent that less than one-tenth of the growth ring is added over this period.

Mathematical expressions of this type are, of course, merely broad generalizations. The very nature of their derivation implies a continuity of improvement and deterioration in growth condition whereby the growth rate increases steadily to a maximum and falls steadily to zero. This occurs relatively rarely, if ever, in nature. The adjustments involved produce inaccuracies which become magnified in the derived curves (as will become apparent later) but the expressions do give a working approximation to the amount of growth which can be expected in the species growing on the site under examination.

TABLE 2
SUMMARY OF MORE OBVIOUS FEATURES OF THE GROWTH CURVES OF *E. GIGANTEA*
AT DIFFERENT LEVELS

Characteristic	Ground Level	Breast Height	Half Height
Growth commences	Last week in September to first week in October	Second week in September	Early September
First tenth of ring completed	Mid October	Second week in October	Mid October
First quarter of ring completed	Early November	Second week in November	Third week in November
Half ring completed	Mid December	End of December	First week in January
Three-quarters of ring completed	Early February	Late February	Late February
Nine-tenths of ring completed	Early April	Mid April	Mid April
Average number of cells cut off in one year	171	205	227

(b) Commencement of Growth

Working with timber species of the northern hemisphere Priestley, Scott, and Malins (1933) noted that resurgence of cambial activity took place first in the crown behind the growing shoots and progressed with variable rapidity, depending on species, down the trunk. In some species the progress of the growth stimulus was very rapid and in others much more protracted. The growth curves in Figure 1 suggest that *E. gigantea* is of the latter type, growth at ground level beginning about three weeks later than at half height. In order to confirm these observations some sample trees were felled every three or four days during the last month (Sept.-Oct. 1946) of the experiment. The first indication of cambial activity for the new growing season was observed on September 6, September 13, and October 1 for half height, breast height, and ground level respectively. The same conclusion may also be drawn from a

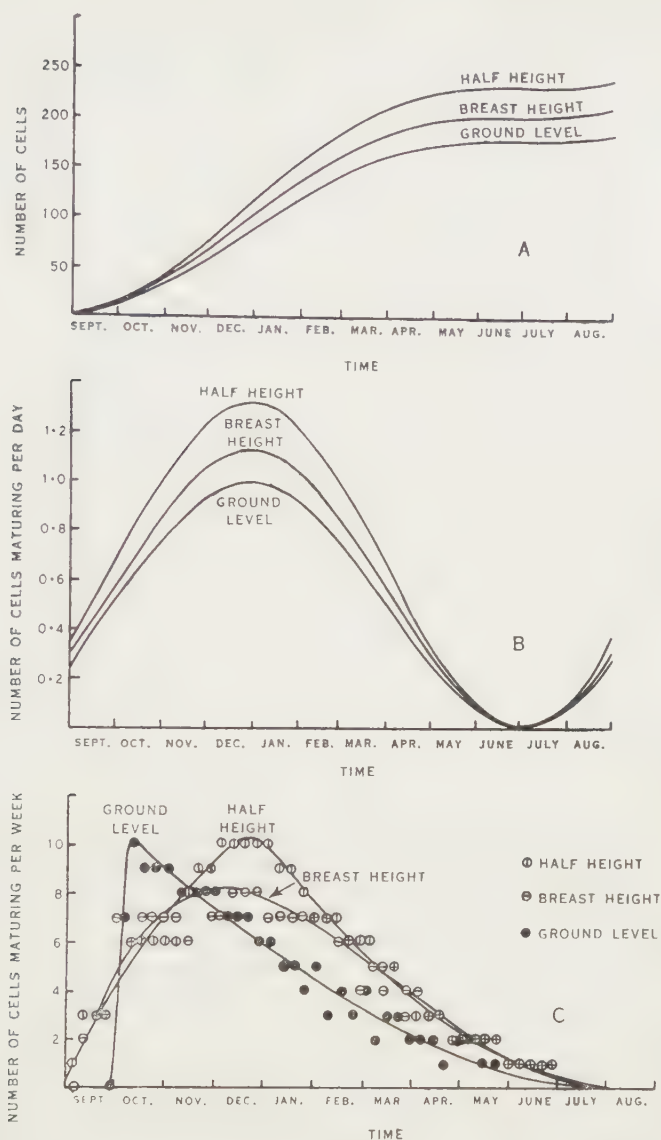


Fig. 2.—Fitted growth curves and growth rate curves, both derived and actual.

(A) Fitted growth curves for the three levels, half height, breast height, and ground level.

$$\text{Half height } y = 86.4 + 0.503x + 28.3 \sin x,$$

$$\text{Breast height } y = 99.5 + 0.569x + 32.6 \sin x,$$

$$\text{Ground level } y = 112 + 0.668x + 38.3 \sin x$$

$$(x = \frac{300}{365} \times \text{no. of days from Jan. 1}).$$

(B) Derived growth rate curves $dy/dx = b + c\pi/180 \cos x$.

(C) Growth rates at various levels determined as a curve of increments.

study of the 1944-45 growth ring in the various trees collected for examination. Specimens from half height invariably showed two bands of abnormal tissue (Plate 2, Figs. 1-3); some specimens from breast height showed two bands and others only one; specimens from ground level showed one band only (Plate 2, Fig. 4) and that not always well developed. The two bands at half height were always about 20 cells apart and one always appeared at the commencement of the growth zone, sometimes after a few fibres had been produced. As the two bands occurred in all trees from the stand it can be accepted that the same causal agent was responsible and therefore conditions conducive to the production of abnormal tissue arose twice during the early period of growth in September and October 1944. One band did not always appear at breast height, indicating that the cambium was still dormant at this level in some trees when this band of abnormal tissue was being produced at half height. At ground level, where only one band (the second in the growth period) appeared, it was always at the beginning of the growth ring, indicating a definite time lag between commencement of growth at half height and ground level sufficient for the production of 20-25 cells at half height.

Initiation of growth is dependent upon the interaction of a number of internal and environmental factors, of which the descent of hormone and nutrients from the crown, the insulating effect of the ground, and photoperiodic factors probably have separate influences. Consideration of such interactions is beyond the scope of this paper but it has been established that it takes a period of 3-4 weeks for conditions suitable for cambial division to progress a distance of some 6-7 ft. down the bole, at least in young saplings.

(c) *Woody Tissue laid down during Early Stages of Growth*

The examination of the material collected from the Australian Capital Territory shows that, upon resumption of cambial activity in the spring, a number of fibres readily distinguishable from the late wood of the previous growth ring are produced (see Plate 1, Fig. 1). Vessels are infrequent, usually entirely absent, in these first-formed cells. The same feature has been observed in numerous specimens of this species from New South Wales, Victoria, and the Australian Capital Territory (see Plate 1, Fig. 4) but not in specimens from Tasmania, which show an obviously different arrangement, the late wood of each growth cycle being poorly defined (Plate 1, Fig. 2). The difference is well illustrated in Figure 3 in which a comparison has been made between the number of fibres produced by the cambium before the first formation of vessels in specimens from the Australian Capital Territory and a number of Tasmanian trees. The data have been summarized in Table 3. From the table and the histograms it appears that the Tasmanian trees belong to a different statistical population from those of the Australian Capital Territory. Not all mainland specimens of *E. gigantea* fall into the latter class; occasionally intermediate types are noted, but the great majority follow the pattern observed for the Australian Capital Territory specimens. From the histograms in Figure 3 it is evident that initial vessel formation in the Australian Capital Territory

specimens is most rapid after some 18-20 fibres have been developed by the cambium, the time of this maximum vessel formation corresponding to about mid October. This is subsequent to the development of approximately one-tenth of the growth ring.

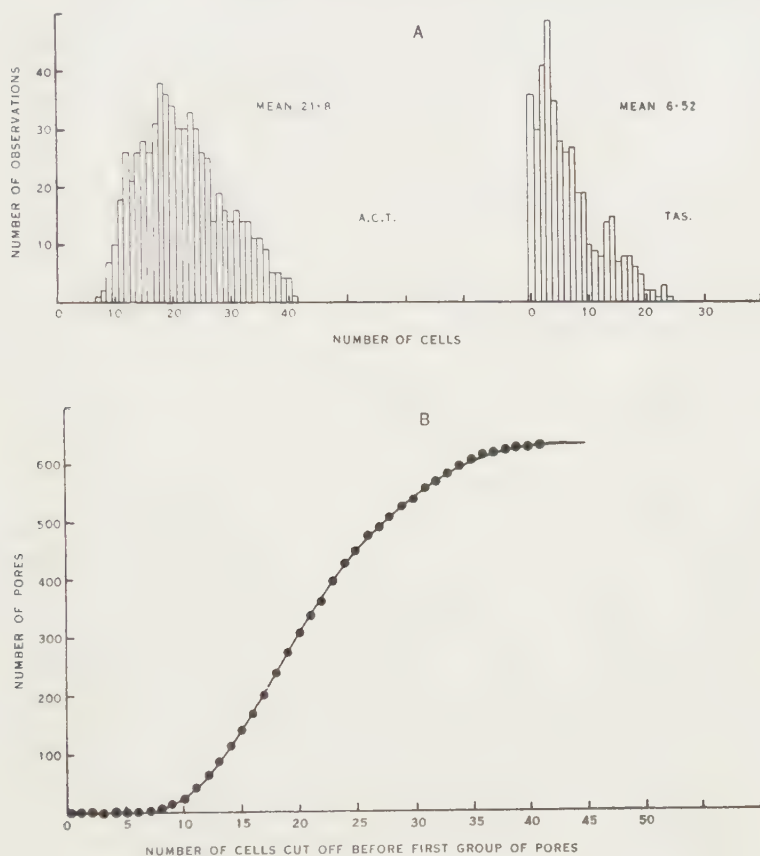


Fig. 3.—Number of fibres cut off by cambial initial before production of first vessel.

- (A) Histograms of Australian Capital Territory and Tasmanian samples compared.
- (B) Cumulative curve of Australian Capital Territory histogram in Figure 2 (A).

The delay in vessel formation at the commencement of growth is, of course, quite distinct from what is observed in northern hemisphere species where the flush of growth in spring is accompanied by the immediate formation of vessels, which, in the typical ring-porous species, are very large in comparison with those formed later in the season. It is of interest to note, however, that in one sample of *Fagus sylvatica* L. examined in this laboratory there was definite evidence of the development of up to five rows of fibres in the first-formed early wood before the development of vessels.

The fact that the vessel pattern in *E. gigantea* differs according to the origin of the specimen, namely whether from Tasmania or the mainland, suggests that climatic and environmental factors are responsible for appreciable variations in anatomical structure. However, the possibility of an inherent varietal difference has not been overlooked, and seed of Australian Capital Territory origin is being grown in Tasmania and vice versa. Examination of the wood structure of the two groups will be made when they reach the sapling stage.

TABLE 3
OCCURRENCE OF FIRST-FORMED VESSELS IN THE EARLY WOOD OF *E. GIGANTEA*

Range (Number of Fibres before First Vessel)	Percentage of First Vessels in Range	
	A.C.T. Material	Tasmanian Material
0- 9	1.6	75.7
10-19	41.3	22.1
20-29	40.0	2.2
30 and over	17.1	Nil

A further observation on the vessel pattern from pith to bark of 15 Australian Capital Territory specimens selected at random suggests a relationship between distance from the crown and number of fibres produced before vessel development. In each growth ring at ground level the average number of fibres produced before the first formation of vessels was determined; the results are summarized in Table 4. It is apparent that the number of fibres maturing before vessels are produced increased with age of the tree. This is also apparent at higher levels and it was observed that in any one growth ring the number of fibres cut off by the cambium before vessel formation decreased from ground level up to half height. It is possible therefore that the crown of the tree is intimately connected with vessel production.

It is fairly generally accepted that resurgence of cambial activity in the stem is associated with the descent of hormone from the crown. From the above evidence it would also appear that the time of vessel production in relation to commencement of growth is also dependent on distance from the crown. Cambial activity, in the trees examined, was initiated at ground level 3-4 weeks later than at half height; delay in vessel production over the same distance may be as much as 6-8 weeks. This phenomenon could be explained on the basis of the supposition that the crown produced two substances, one initiating cambial division and the other stimulating vessel production, the latter descending from the crown at a slower rate than the former. The bands of vessel-free (or comparatively so) late wood in mainland specimens of *E. gigantea* would result from the inactivation of one of these stimuli and cessation of growth from the inactivation of the other. Late production of vessels in those portions of the woody stem removed from the crown would be repeated in each growth

cycle. It is possible that the stimulus affecting vessel production is more sensitive to climatic and environmental changes than the growth stimulus. In those trees, particularly from Tasmania, where it is assumed that growth does not entirely cease, as evidenced by indistinct growth rings, there would not be complete inactivation of either stimulus, and both growth and vessel development would be continuous.

TABLE 4

NUMBER OF FIBRES PRODUCED IN GROWTH RINGS OF *E. GIGANTEA* FROM THE AUSTRALIAN CAPITAL TERRITORY AT GROUND LEVEL BEFORE FIRST VESSELS

Ring	Range	Average
1934-35	0	0
1935-36	0-1	0
1936-37	0-4	1
1937-38	1-10	3
1938-39	1-16	4
1939-40	3-11	5
1940-41	3-11	5
1941-42	4-16	9
1942-43	6-24	13
1943-44	6-27	14
1944-45	9-42	21
1945-46	11-43	22

(d) Variation in Growth Rate

Growth rates have been expressed throughout the growing season as curves derived from the equations of the form $y = a + bx + c \sin x$ fitted to the cell count data (see Fig. 2B) and also as curves of increments from the actual growth curves (see Fig. 2C). As noted earlier, many of the small differences between growth at the different levels and the more subtle growth characters that are intimately related to the structure of the wood are masked by the mathematical treatment. Substituting the known values of b and c in the equation $dy/dx = b + c\pi/180 \cos x$, maximum growth rates on January 1 may be calculated. These are 0.99, 1.12, and 1.32 cells per day for ground level, breast height, and half height respectively.

The curves of increments, however, show variations in the dates of maximum growth rates although their magnitudes are not appreciably different from the calculated values. Growth begins at ground level 3-4 weeks later than at half height but the maximum growth rate of about 10 cells per week is reached within a fortnight and thereafter falls off slowly over the remainder of the growing season. On the other hand, the growth at half height increases more slowly, taking about four months to reach a maximum, also of about 10 cells per week, at which time the growth rate at ground level has been reduced to $3/5$ of its maximum. From midsummer onwards the growth rate at all levels falls off over the remainder of the growth period, reaching zero in late winter. In general the resurgence of cambial activity occurs sooner nearer the crown but the achievement of maximum growth rate is most rapid near the bottom of the stem.

(e) Late Wood Formation and Dormancy

The first sign of late wood formation in the Australian Capital Territory specimens, i.e. thickening of the cell wall followed by a reduction in the radial diameter of the cell, takes place towards the end of May or beginning of June since the trees felled in mid June showed a little late wood formation.

One of the original purposes of this study was to determine whether or not *E. gigantea* growing in the Australian Capital Territory passes through a dormant stage, and, if so, to estimate the length of this period. In late August,

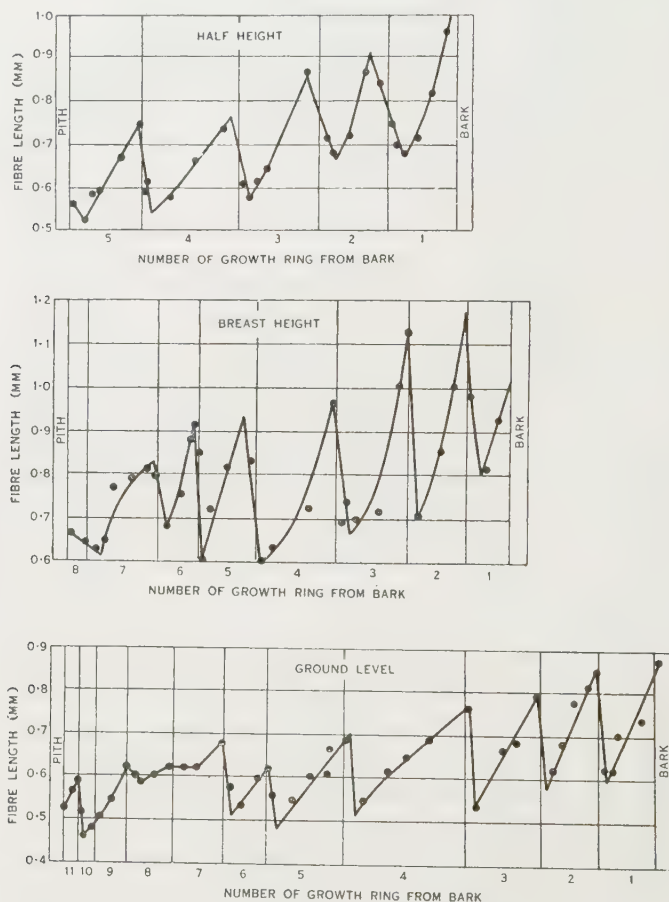


Fig. 4.—Variation of fibre length with position in growth ring, height in tree, and number of growth ring from the bark in *Eucalyptus gigantea*.

for a few weeks until the resurgence of cambial activity, the growth rate has reached a very low level. This period may therefore be considered as constituting a dormant period. The absence of the first band of abnormal tissue from the ground level specimens in the 1944-45 ring is confirmatory evidence of at least a short period of dormancy between the last-formed late wood and

the resurgence of growth and this period may extend well into the spring at the lower levels in the stem.

(f) *Fibre Dimensions and Growth Rate*

(i) *Variation in Fibre Length.*—The results of fibre length variation at three levels within the one tree are presented graphically in Figure 4. In nearly all growth rings irrespective of height there is an increase in fibre length from early wood to late wood followed by a sudden drop in passing to the early wood of the next ring. This observation has been extended to other angiosperm woods, and has been found to be a principle generally applicable to woods with distinct growth rings (Bisset, Dadswell, and Amos 1950). Some

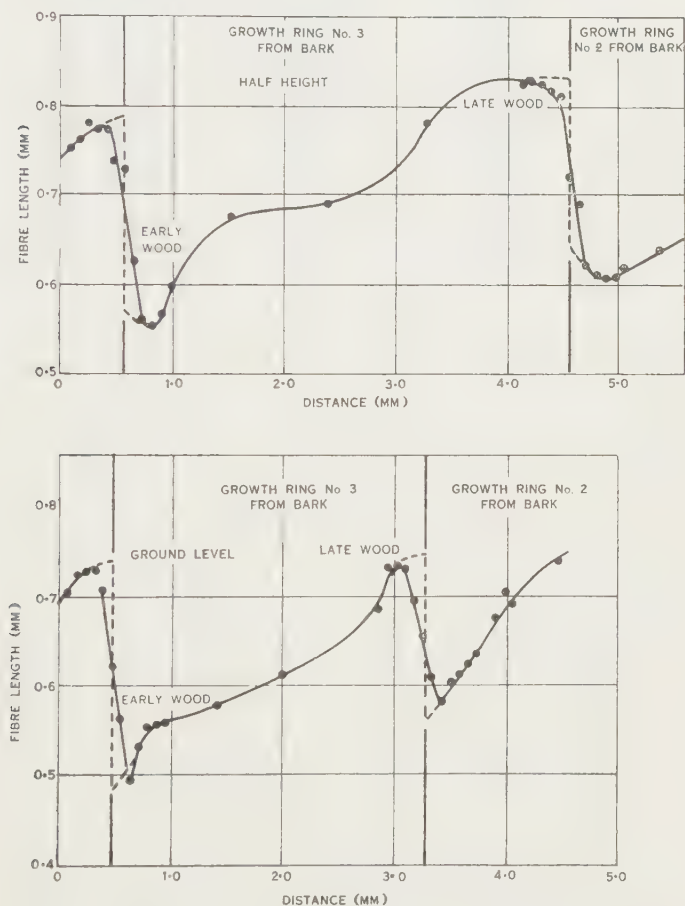


Fig. 5.—Variation of fibre length within a single growth ring of *Eucalyptus gigantea* at half height and ground level.

irregularities were apparent near the pith at ground level but in the outer rings, where positioning and general technique were of a high degree of accuracy, the phenomenon is most striking.

The 1942-43 growth ring was selected for more detailed study. Results of fibre length measurements at various positions in this ring, both at ground level and half height, are shown in Figure 5. It is fairly obvious that there is, in fact, a very sudden drop in length from the last-formed late wood of one growth ring to the first-formed early wood of the next. Although in Figure 5 results of fibre measurements in the region of the junction between late wood and early wood indicate a gradual drop it is a matter of simple mathematics

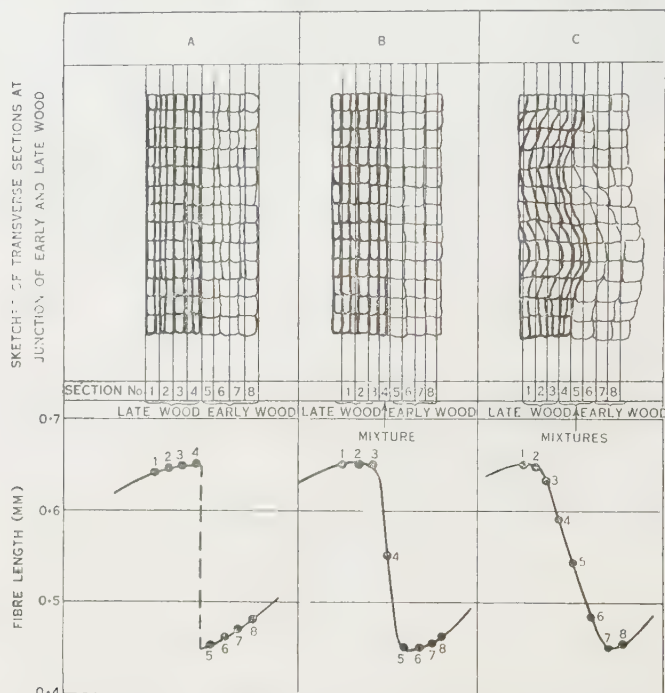


Fig. 6.—Effect of curvature of late wood-early wood junction on the shape of the fibre length distribution curve.

- (A) Straight early wood-late wood junction. Tangential longitudinal late wood sections completely distinct from early wood sections, giving rise to "discontinuity" in fibre length distribution curve.
- (B) Straight early wood-late wood junction but section No. 4 contains both early and late wood fibres; hence intermediate value on sloping fibre length curve.
- (C) Irregular (common) early wood-late wood junction causing several sections to contain varying proportions of late and early wood fibres and giving rise to an apparent gradual decrease in fibre length from late wood to early wood.

to demonstrate that the actual results obtained and plotted are due partly to the fact that the junction between late wood and early wood is never a straight line and partly to the thickness of the sections employed for maceration. This is shown in Figure 6 which is self-explanatory.

The results obtained for the 1942-43 growth ring have, in Figure 7, been plotted against time during the growing season, the position-time transposition being obtained from Figure 1. This transposition is important for correlation of fibre lengths with growth rate and for such a comparison the growth rate curves have been added to Figure 7. It is immediately apparent that at the period of maximum growth rate the shortest fibres are developed and conversely that at the period of minimum growth rate the longest fibres are formed. This opposed relationship is particularly striking at ground level. At half height

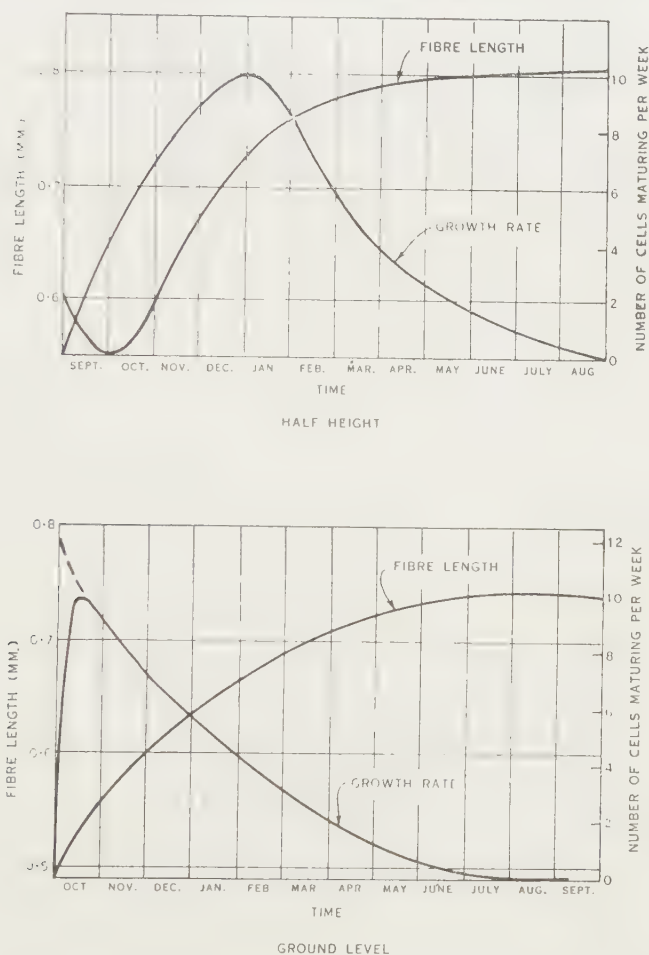


Fig. 7.—Comparison of growth rate and fibre length. (Fibre length data from Figure 5 with abscissae adjusted so that equal intervals represent equal time intervals.)

the same general features are observed although the positions of maximum growth rate and minimum fibre length are not nearly so coincident. Variation of this type can be expected where average data are compared with individual observations.

The accommodation of fibres of varying length within the one growth ring can be accounted for to some extent by the variation in fibre overlap from early wood to late wood. Actual observation has shown that the degree of overlap can be measured and it was found that the amount of fibre involved in such overlap was approximately twice as great in late wood as in early wood fibres. Fibres from first-formed early wood and last-formed late wood of the one growth ring are depicted in Plate 3, Figures 3 and 4.

(ii) *Variation in Fibre Diameter.*—Results of measurements of the radial diameters of fibres through the 1942-43 growth ring at three levels in the one tree are shown graphically in Figure 8. During the later part of the growth period the radial diameters progressively decrease, reaching a minimum in the late wood. Resurgence of growth after the dormant period is accompanied by a sudden increase in radial diameter. The curves for fibre diameter variation at the three levels show a striking similarity to the growth rate curves (see Figure 2C) and it is apparent that where growth is rapid fibre diameter is at a maximum and where growth is slow fibre diameter is at a minimum. It is also apparent that the shortest fibres have the greatest diameter and the longest fibres the smallest diameter.

At half height the fibres do not reach their maximum radial diameter until some time after the commencement of new growth in the spring, whereas at ground level maximum diameter coincides with resurgence of cambial activity. This agrees with the observed variation in growth rate. In photomicrographs of the 1942-43 growth ring taken at ground level and half height (Plate 3, Figs. 1 and 2) it is evident that at ground level, the first-formed fibres have the largest radial diameters, but at half height the first-formed fibres are somewhat thicker-walled and smaller than those formed subsequently.

(iii) *General.*—The results reported provide a further example of the principle so ably expounded by Thompson (1942), namely that growth and form are intimately connected and that one can frequently be deduced if the other is known. In the present case it appears that the growing conditions greatly influence the structure of the organism and it is of interest to note the magnitude of the variation in fibre dimensions, one of which, the radial diameter, changes with the growth rate, and the other, the length, changes in opposition to it. The variations have been summarized in Figure 9 in which a photomicrograph of the same growth ring has been included for comparison. From this figure it is apparent that, if the minimum length of the fibres within a ring be designated by L , the maximum length is approximately $3L/2$; from Figures 1 and 2 it is also apparent that, if the minimum radial diameter be designated by r , the maximum is approximately $9r/4 = (3/2)^2 r$. Since the tangential diameter of the fibres is nearly constant the indication is that, within a particular growth cycle, the volumes of the maturing elements vary inversely as their lengths, i.e. directly as the growth rate.

The outcome of the study of fibre dimension in this species has been to elucidate a principle of growth in woody angiosperms which appears to be of general application, and to complete a pattern of growth investigated many

years ago by Sanio (1872) who found that average fibre lengths in successive growth rings showed an initial increase, the increase decreasing in an exponential manner from year to year. Superimposed upon this overall variation is an

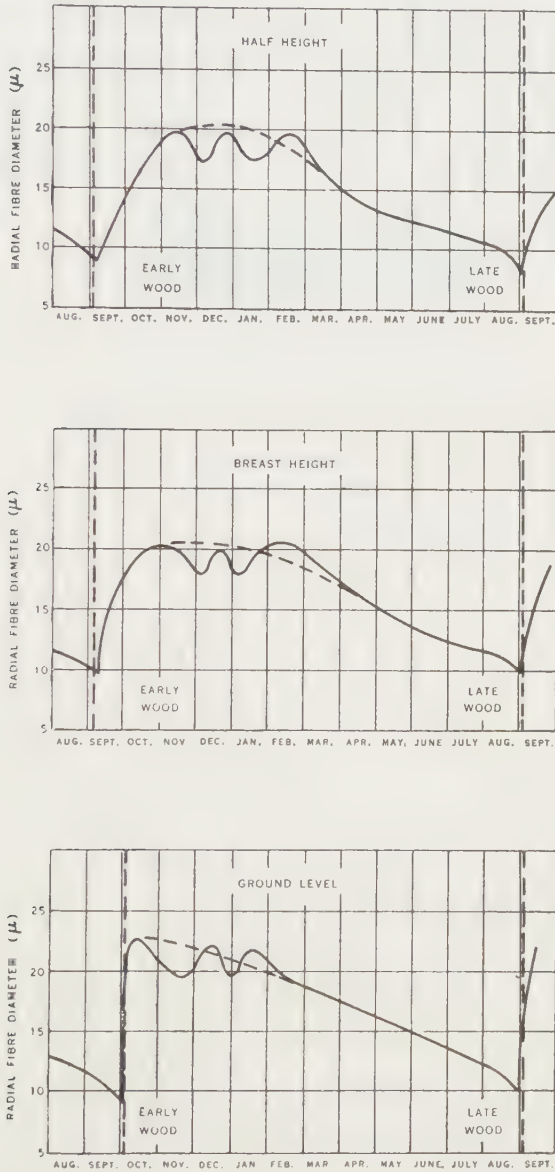


Fig. 8.—Variation in fibre diameter within the same growth ring at various levels in the tree. The curves shown --- smooth over small variations during the growth period that have no general significance.

annual variation which is intimately connected with the variation in growth rate during the seasonal growing periods.

(g) *Anomalous Tissue*

As previously mentioned, the two bands of anomalous tissue occurring at half height in all trees from the area are characteristic for the 1944-45 growth ring and serve as an identification feature for the ring. Microscopic examination of the tissue revealed that the fibrous cells produced were tangentially expanded and that the ray cells had proliferated and were frequently divided by vertical walls parallel to the direction of the ray. Large numbers of more or less isodiametric cells were produced, as illustrated in Plate 2. Vessels between the two bands were completely tylosed and without function.

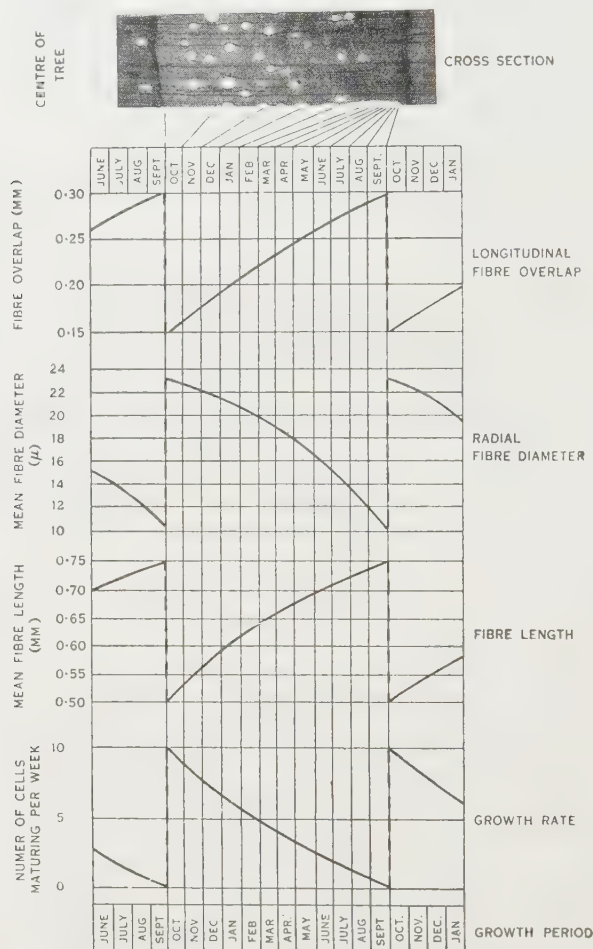


Fig. 9.—Idealized correlations of growth rate and fibre dimensions at ground level in *Eucalyptus gigantea*.

In an effort to determine the reasons for the formation of this tissue climatic data for the area were obtained. These data have been summarized in Table 5, from which it will be apparent that, at the time the tissues were formed, namely early in the 1944-45 growing season, the saturation deficit and

temperature were higher than normal and the rainfall lower. Furthermore, these conditions followed a long, dry winter and were more severe than had been experienced in the previous six years for which data were available. The temperatures, although high for this period of the year, were not as high as summer temperatures. It is, however, probable that the lack of available moisture after the dry winter and the unusually high saturation deficit were responsible for overtaxing the water balance within the trees at a time when the young shoots were developing. It would therefore appear that the tissues were laid down under conditions corresponding to drought when the trees were relatively poorly equipped to resist a severe climate. The general occurrence of the two bands of this tissue in the young trees of *E. gigantea* from the area specified is a valuable reference point for any further study of trees from the same area.

TABLE 5
CLIMATIC DATA LATE AUGUST TO MID OCTOBER
Summary for Years 1939-1944

Period	Saturation Deficit (in. Hg)		Rainfall (points)		Temperature (Max.) (°F.)	
	Average		Average		Average	
	1944	1939-1943	1944	1939-1943	1944	1939-1943
26.viii.-2.ix.	0.33*	0.20	2	52	65*	62
2.ix.-9.ix.	0.42*	0.24	0†	58	70	68
9.ix.-16.ix.	0.24	0.25	15	62	65	69
16.ix.-23.ix.	0.43*	0.25	0†	90	72	70
23.ix.-30.ix.	0.42*	0.29	30	25	78*	72
30.ix.-7.x.	0.41	0.34	9	61	78*	76
7.x.-14.x.	0.82*	0.33	0†	89	87*	71
14.x.-21.x.	0.51*	0.38	—	—	88*	76
Mean	0.45	0.26	8	62	75	68

* Highest value recorded for the period in any year.
† Lowest value recorded for the period in any year.

V. CONCLUSIONS

(i) Growth of *Eucalyptus gigantea* Hook. f. in the Australian Capital Territory continues well into the winter months, definite late wood formation beginning as late as the end of May.

(ii) There was a comparatively short period of dormancy at the end of the growing period and before the recommencement of growth in the early spring (beginning of September) in the Australian Capital Territory trees examined.

(iii) Resurgence of growth began in all trees at the higher levels and was not observed at ground level until 3-4 weeks after its inception at half height.

(iv) The growth rates varied at different levels; the maximum growth rate for the period under observation was reached at ground level just a few

days after inception of growth for the season although at half height the maximum was not reached until several months had passed.

(v) The comparative absence of vessels from the first-formed early wood is a feature of the mainland trees of this species; in the specimens examined it was a particularly noticeable feature of the outer rings at ground level, but less obvious at higher levels and in rings close to the centre of the tree.

(vi) It has been suggested that this characteristic feature of late production of vessels after commencement of growth is related to distance from the crown and a possible mechanism has been discussed.

(vii) The Tasmanian specimens examined did not show either distinct growth rings or a vessel-free zone in the first-formed early wood; this difference from the majority of mainland specimens has been ascribed tentatively to variations in climate and environment rather than to any varietal difference.

(viii) It was continually found that the fibres of the early wood were shorter than those of the late wood and that there was a sudden decrease in fibre length from the late wood of one growth ring to the first-formed early wood of the next; on the other hand, the early wood fibres were found to have a consistently larger radial diameter than the late wood fibres. Thus there was a definite correlation between growth rate and fibre dimensions, the shortest, widest fibres of any one growth ring were laid down at the period of maximum growth rate and conversely the longest, narrowest fibres were laid down at the period of minimum growth rate. It has been shown that the greater length of the late wood fibres is accommodated by the greater amount of fibre overlap.

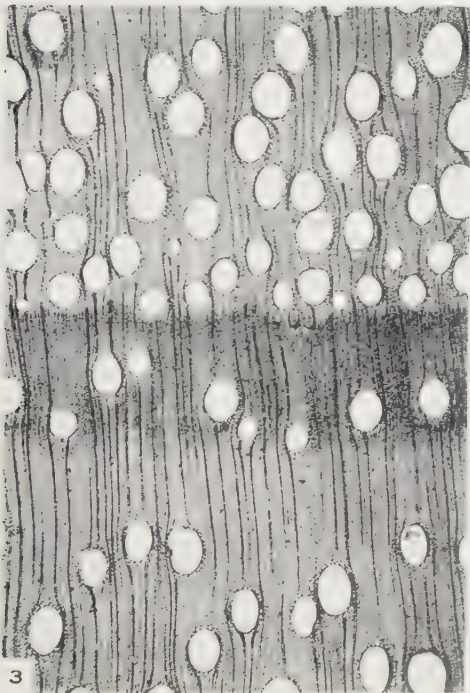
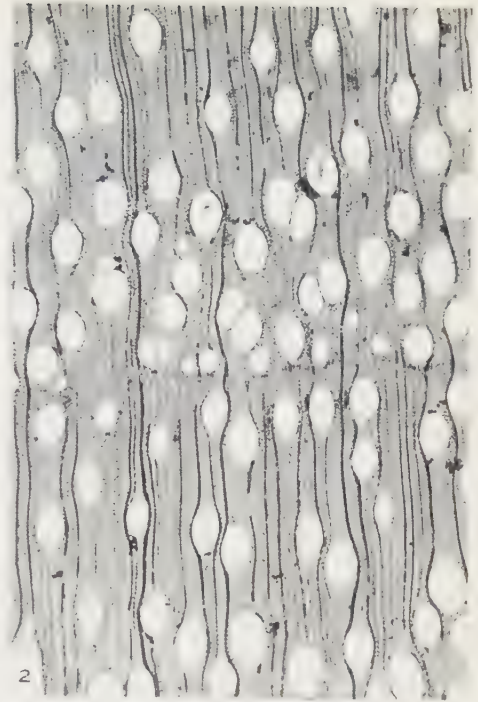
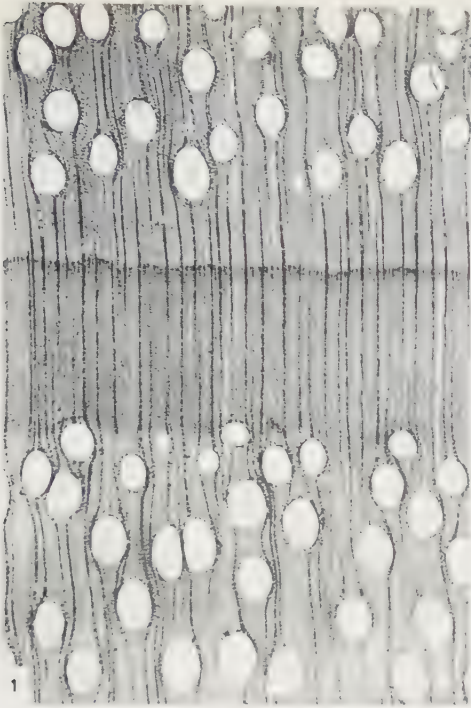
(ix) A relationship has been observed between the formation of certain anomalous tissue during the early portion of the 1944-45 growing period and climatic conditions at the time; it has been suggested that this particular tissue was formed in response to severe drought conditions.

VI: ACKNOWLEDGMENTS

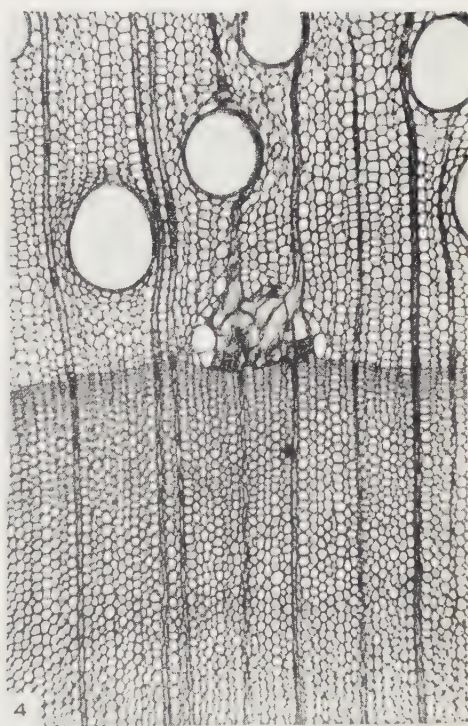
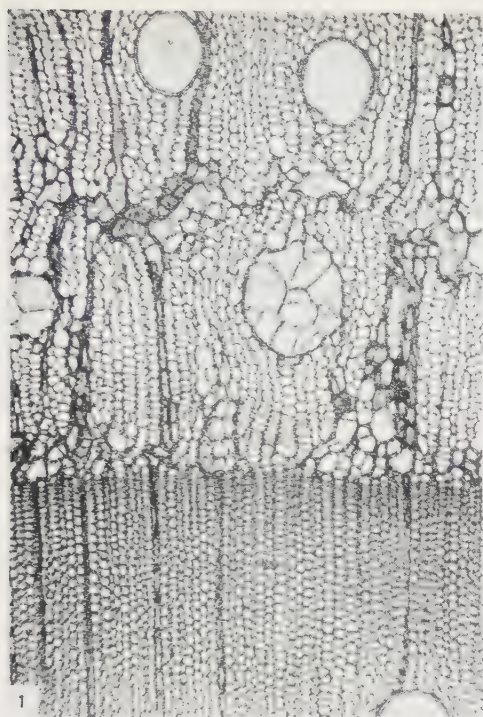
The authors are indebted to Misses M. Murphy, J. Hider-Smith, W. Matthews, and L. Wills for their painstaking work in the preparation of sections and fibre measurements necessary for the study, and to Mr. E. Williams for statistical assistance. The collection of the material from the Australian Capital Territory by Dr. M. Jacobs is also gratefully acknowledged.

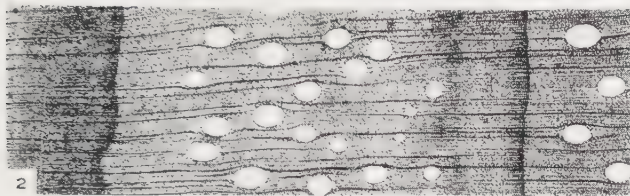
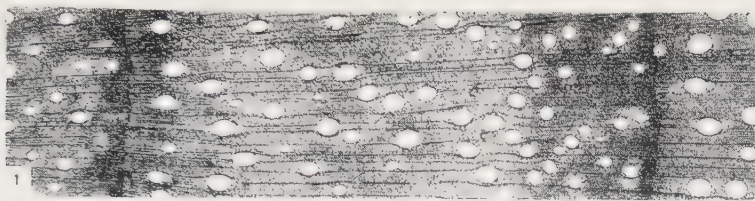
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AMOS, BISSET, AND DADSWELL.—WOOD STRUCTURE IN RELATION TO GROWTH IN
EUCALYPTUS GIGANTEA HOOK. F.





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EXPLANATION OF PLATES 1-3

PLATE 1

- Fig. 1.—*Eucalyptus gigantea*, grown in Australian Capital Territory, showing band of late wood and early wood without vessels. x 25.
- Fig. 2.—*E. gigantea*, grown in Tasmania, showing narrow and indistinct late wood with vessels formed early in the early wood. x 25.
- Fig. 3.—*E. gigantea*, intermediate type, sometimes found in mainland specimens, showing distinct band of late wood well supplied with vessels, and a new crop of small vessels formed in the early wood. The vessels become larger in the later early wood. x 25.
- Fig. 4.—*E. gigantea*, grown in Victoria, showing the band of early wood without vessels and distinct growth check at the end of late wood formation. x 120.

PLATE 2

- Fig. 1.—Transverse section of *E. gigantea*, half height, grown in the Australian Capital Territory, showing two bands of abnormal tissue at the commencement of the 1944-45 growth ring and about 25 cells later. Vessels tylosed between the bands. x 100.
- Fig. 2.—Longitudinal radial section of *E. gigantea*, half height, grown in Australian Capital Territory, showing two bands of abnormal tissue. x 100.
- Fig. 3.—As for Figure 2, showing two bands of abnormal tissue, with normal vessel and fibres developed further out. x 50.
- Fig. 4.—Transverse section of same tree at ground level showing small amount of abnormal tissue corresponding to the second band in Figure 1. x 100.

PLATE 3

- Figs. 1 and 2.—The different *initial* rates of growth in the third annual growth ring from the bark in a tree of *Eucalyptus gigantea* at half height and ground level are shown by the smaller radial diameter of the fibres and their darker appearance at half height (Fig. 1) compared with the more open texture of the ring at ground level (Fig. 2). There is also a larger number of vessels and fibres at half height. The number of fibres formed before vessels are formed is markedly different at the two levels.
- Fig. 3.—Early wood fibres.
- Fig. 4.—Late wood fibres of the same growth ring as Figure 3. The photomicrographs show the short, stubby nature of early wood fibres and the long, tapering character of late wood fibres. The extent of overlap is indicated by the rather abrupt shoulders near the end of the early wood fibres. In the late wood fibres these shoulders are much further from the ends and are more sloping.

MOLYBDENUM IN NITROGEN METABOLISM OF LEGUMES AND NON-LEGUMES

By A. J. ANDERSON* and D. SPENCER*

[Manuscript received June 7, 1950]

Summary

A study was made of the growth and nitrogen metabolism of plants in relation to molybdenum supply. Four soils low in available molybdenum were used in pot cultures for this work.

Subterranean clover (*Trifolium subterraneum* L.) responded to molybdenum on each of the soils. Flax (*Linum usitatissimum* L.) responded to molybdenum on only one of the soils in the presence of a heavy dressing of manganese sulphate, which reduced the uptake of molybdenum and induced molybdenum deficiency. Oats (*Avena sativa* L.) did not respond to molybdenum on any of the soils, even where manganese sulphate was added.

Deficiency of molybdenum decreased the percentage protein nitrogen in the flax and in the clover, but in different ways. Molybdenum increased the yield of flax only where combined nitrogen, either as nitric acid or ammonium sulphate, was provided. High nitrate accumulation occurred in the flax provided with nitric acid but without molybdenum. The percentage total nitrogen in flax was unaffected by molybdenum, but deficiency of molybdenum decreased the percentage protein nitrogen and increased the percentage non-protein nitrogen. Thus molybdenum was needed by the non-legume for the utilization of absorbed nitrate nitrogen.

Molybdenum markedly increased both the yield and percentage nitrogen of clover where no combined nitrogen was provided, but had little or no effect where nitrogen was supplied as nitric acid or ammonium sulphate. This was despite the fact that each of these nitrogen compounds decreased the uptake of molybdenum by the clover. Deficiency of molybdenum decreased both the percentage protein nitrogen and the percentage non-protein nitrogen in the clover. The evidence with clover shows that molybdenum is directly concerned in symbiotic nitrogen fixation, and that the molybdenum requirement for optimum symbiotic nitrogen fixation is appreciably greater than the requirement for optimum utilization of nitrate nitrogen.

I. INTRODUCTION

In an earlier paper (Anderson and Thomas 1946) it was shown that symbiotic nitrogen fixation in legumes is seriously inhibited by molybdenum deficiency. The legumes were well nodulated but were stunted and contained a low percentage of nitrogen. Molybdenum-deficient clover responded normally to applications of combined nitrogen compounds, and when combined nitrogen was thus supplied there was no response to molybdenum. When ammonium sulphate was used as a source of nitrogen, it reduced the pH of the soil and

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greatly reduced the uptake of molybdenum, but even under these conditions no response to molybdenum occurred. The results indicated that molybdenum is directly concerned in nitrogen fixation.

Nitrogen applied as sodium nitrate increased the growth of the legumes as did molybdenum or ammonium sulphate. However, sodium nitrate increased the pH of the soil and increased the uptake of molybdenum, and it is therefore possible that molybdenum released from the soil contributed in part to the effect of this nitrogen compound.

Evidence obtained with the microorganisms *Aspergillus* and *Azotobacter* indicates that more molybdenum is needed for the utilization of nitrate than of ammonium nitrogen (Steinberg 1937, 1939; Bortels 1936; Mulder 1948). The present experiments were carried out to examine the effect of nitrate nitrogen on the response of plants to molybdenum, and to compare the effects of molybdenum on the nitrogen metabolism of legumes and non-legumes.

II. EXPERIMENTAL

(a) General

Plants were grown in an unheated glasshouse on soils from the Southern Tablelands of New South Wales, previously shown to be deficient in molybdenum (Anderson 1948). The Bacchus Marsh strain of *Trifolium subterraneum* L. was the legume used. The non-legumes used were flax (*Linum usitatissimum* L.) and Algerian oats (*Avena sativa* L.). The clover seed was inoculated with an effective strain of *Rhizobium* immediately before sowing. Ten plants were grown in each glazed earthenware pot; five plants in each glass pot in Experiment 10; and fifteen plants in each enamel pot in Experiment 11.

All plants were harvested before flowering or when the first few flowers had formed. The plant material was dried rapidly under forced draught at 50°C.

Nutrients other than nitrogen were applied in solution at the beginning of each trial. Nitrogen fertilizers were applied in small successive doses, the nitric acid in solutions containing up to 0.2 per cent. nitric acid. The dressings applied are expressed as cwt. per acre based on the surface area of the pot, and the equivalents in milligrams per pot are given for each treatment. Randomization of treatments was adopted throughout.

Weights of oven-dry soil in each pot are given in the details of particular experiments. The pots were weighed frequently and the soils watered to field capacity. The soils used were all of more acid reaction than pH 5.5. They are identified in the text as:

Soil A.—a grey-brown loam from the Dickson Experiment Station, C.S.I.R.O., Canberra.

Soil B.—a light, yellow-grey, sandy loam of sedimentary origin, from Kingsdale, near Goulburn, N.S.W.

Soil C.—a brown to reddish brown loam of basaltic origin from Roslyn, N.S.W.

Soil D.—a grey to light grey, loamy, coarse sand of granitic origin from Carrick, N.S.W.

The transformation values used in the statistical analysis of the data to secure uniformity of variance within treatments were the square root transformation for dry matter (Tables 8 and 9), log transformations of the form $A + \log(x + B)$ for the data on nodulation, and log transformations of the form $A + \log x$ for the data on chemical analysis. The constant A has been introduced into these transformations primarily to avoid negative values.

(b) Details of Experiments

Experiment 1.—To test the effect of molybdenum on subterranean clover, at different levels of application of ammonium sulphate and manganese sulphate. *Soil A.* Sown on June 3, 1948, and harvested on October 7. A $2 \times 2 \times 2$ factorial combination of the following fertilizers was used:

Sodium molybdate — nil or 1 lb. per acre ($\equiv 1.21$ mg. per pot).

Ammonium sulphate — nil or 7 cwt. per acre ($\equiv 952$ mg. per pot).

Manganese sulphate — nil or 5 cwt. per acre ($\equiv 680$ mg. per pot).

Replications: 2. *Soil:* 1.51 Kg. of soil *A* in glazed earthenware pots, with 3 cwt. of monocalcium phosphate per acre on all pots.

Experiment 2.—Details as for Experiment 1. *Soil B.* (1.48 Kg. soil per pot —harvested on September 21).

Experiment 3.—Details as for Experiment 1. *Soil C.* (1.22 Kg. soil per pot).

Experiment 4.—To test the effect of molybdenum on flax, at different levels of application of ammonium sulphate, nitric acid, and manganese sulphate. *Soil B.* Sown on June 3, 1948, and harvested on October 21. A $2 \times 3 \times 2$ factorial combination of the following fertilizers was used:

Sodium molybdate — nil or 1 lb. per acre ($\equiv 1.21$ mg. per pot).

Nitrogen treatments — nil, 6.25 cwt. ammonium sulphate per acre ($\equiv 180$ mg. N per pot), or 180 mg. N per pot applied as nitric acid.

Manganese sulphate — nil or 5 cwt. per acre ($\equiv 680$ mg. per pot).

Replications: 2. *Soil:* 1.47 Kg. of soil *B* in glazed earthenware pots, with 2 cwt. of monocalcium phosphate per acre on all pots.

Experiment 5.—Details as for Experiment 4, on oats.

Experiment 6.—To test the effect of molybdenum, at different levels of manganese sulphate and potassium sulphate, on oats on soil *A* and on flax on soil *C.* Sown on June 3, 1948, and harvested on October 21. A $2 \times 2 \times 2$ factorial combination of the following fertilizers was used:

Sodium molybdate — nil or 1 lb. per acre ($\equiv 1.21$ mg. per pot).

Manganese sulphate — nil or 5 cwt. per acre ($\equiv$ 680 mg. per pot).

Potassium sulphate — nil or 1 cwt. per acre ($\equiv$ 136 mg. per pot) applied 20 days before harvest.

Replications: 1. Soil: 1.51 Kg. of soil *A*, or 1.22 Kg. of soil *C* in glazed earthenware pots, with 2 cwt. of monocalcium phosphate per acre + 180 mg. N per pot applied as nitric acid.

Experiment 7.—Details as for Experiment 6, but with flax on soil *A* and oats on soil *C*, and using 1 cwt. of manganese chloride per acre where potassium was used in Experiment 6.

Experiment 8.—To test the effect of molybdenum on oats, at different levels of application of manganese sulphate. Soil *D*. Sown on June 3, 1948, and harvested on October 21. A 2×2 factorial combination of the following fertilizers was used:

Sodium molybdate — nil or 1 lb. per acre ($\equiv$ 1.21 mg. per pot).

Manganese sulphate — nil or 5 cwt. per acre ($\equiv$ 680 mg. per pot).

Replications: 2. Soil: 1.73 Kg. of soil *D* in glazed earthenware pots, with 2 cwt. of monocalcium phosphate per acre + 2 cwt. of potassium sulphate per acre + 180 mg. N per pot applied as nitric acid.

Experiment 9.—To test the effect of molybdenum on subterranean clover, at different levels of application of nitric acid and manganese sulphate. Soil *D*. Sown on June 3, 1948, and harvested on October 7. A $4 \times 2 \times 2$ factorial combination of the following fertilizers was used:

Sodium molybdate — nil, 1/16 oz. per acre ($\equiv$ 4.7 μ g. per pot), 1 oz. per acre ($\equiv$ 76 μ g. per pot), or 1 lb. per acre ($\equiv$ 1215 μ g. per pot).

Nitric acid — nil or 159 mg. N per pot.

Manganese sulphate — nil or 5 cwt. per acre ($\equiv$ 680 mg. per pot).

Replications: 2. Soil: 1.73 Kg. of soil *D* in glazed earthenware pots, with 3 cwt. of monocalcium phosphate per acre + 2 cwt. of potassium sulphate per acre on all pots.

Experiment 10.—To test the effects of molybdenum, ammonium sulphate, and nitric acid on subterranean clover at different stages of growth. Soil *B*. Sown on June 29, 1948. A 2×2 factorial combination of the following fertilizers was used for the first harvest on September 1:

Sodium molybdate — nil or 2 oz. per acre ($\equiv$ 75 μ g. per pot).

Ammonium sulphate — nil or dressings of 0.75 cwt. per acre ($\equiv$ 10 mg. N per pot) on July 30 and August 6, then 20 mg. N per pot on August 13 and 24.

The above four fertilizer treatments were also used on pots for the second harvest on September 13 with an additional dressing of 20 mg. N per pot as ammonium sulphate applied on September 3; and on pots for the third harvest

on October 11, with additional dressings of 20 mg. N per pot as ammonium sulphate on September 3 and on October 10.

The design for the third harvest also included a 2×2 factorial combination of:

Nitric acid — nil, or 10 mg. N per pot applied on July 30, August 6, 13, 20, and 27, September 3, 9, 16, and 24, and October 1 and 8.

Sodium molybdate — nil, or 2 oz. per acre ($\equiv 75 \mu\text{g. per pot}$).

Replications: 2. Soil: 0.80 Kg. of soil *B* in glass pots, with 2 cwt. of monocalcium phosphate per acre on all pots.

Experiment 11.—To test the effects of molybdenum and nitric acid on subterranean clover and oats. Soils *A* and *B*. Sown on July 2, 1948, and harvested on November 1. A $2 \times 2 \times 2 \times 2$ factorial combination as follows was used:

Sodium molybdate — nil or 4 oz. per acre ($\equiv 696 \mu\text{g. per pot}$).

Nitric acid — nil or 330 mg. N per pot).

Two species — subterranean clover in one set of pots; oats in the other.

Two soils — one set using soil *A*; the other using soil *B*.

Replications: 2. Soil: 4.40 Kg. of soils *A* and *B* in enamel pots, with 2 cwt. of monocalcium phosphate per acre on all pots.

Experiment 12.—To test the effect of molybdenum on subterranean clover, at different levels of application of nitric acid and sodium sulphate. Soil *B*. Sown on June 14, 1949, and harvested on October 13. A $2 \times 2 \times 2$ factorial combination of the following fertilizers was used:

Sodium molybdate — nil or 2 oz. per acre ($\equiv 0.152 \text{ mg. per pot}$).

Two levels of nitric acid — nil or 14.4 mg. N per pot on July 13, 22, and 28, August 5 and 11, and September 1, 8, and 15, then 28.8 mg. N per pot on September 22 and 29.

Sodium sulphate—nil or 1 cwt. per acre ($\equiv 136 \text{ mg. per pot}$).

Replications: 4. Soil: 1.64 Kg. of soil *B* in glazed earthenware pots, with 2 cwt. of monocalcium phosphate per acre + 2 cwt. of potassium chloride per acre.

(c) *Analytical Methods*

Plant tops which were dried rapidly at 50°C. immediately after harvest, were then finely ground, further dried at 101°C. , and cooled in a desiccator prior to chemical analysis.

Total nitrogen was determined on 0.2 g. samples by the Kjeldahl procedure using selenium as a catalyst. The final distillation step was carried out in a Parnas-Wagner steam distillation unit using a 2 per cent. boric acid solution to trap the ammonia as described by Ma and Zuazaga (1942).

Protein nitrogen.—The protein was rendered insoluble by treatment with boiling alcohol, and non-protein nitrogen constituents were extracted in alcohol and in water by the following method: Samples of 0.2-0.4 g. are extracted twice with 25 ml. aliquots of redistilled ethyl alcohol and twice with 25 ml. aliquots of water on a boiling water bath. Each extraction is carried on for 30 minutes. The supernatant after each extraction is filtered through a filter paper disc. The plant residue plus the filter paper disc are washed into a Kjeldahl flask and nitrogen estimated as described for total nitrogen.

Non-protein nitrogen was calculated by difference between the total nitrogen and protein nitrogen fractions.

Molybdenum was estimated according to the method of Dick and Bingley (1947) in which a coloured molybdenum thiocyanate complex is developed, extracted into isoamyl alcohol, and the percentage transmission at $465\ \mu$ measured in a Coleman Universal spectrophotometer and compared with standard solutions.

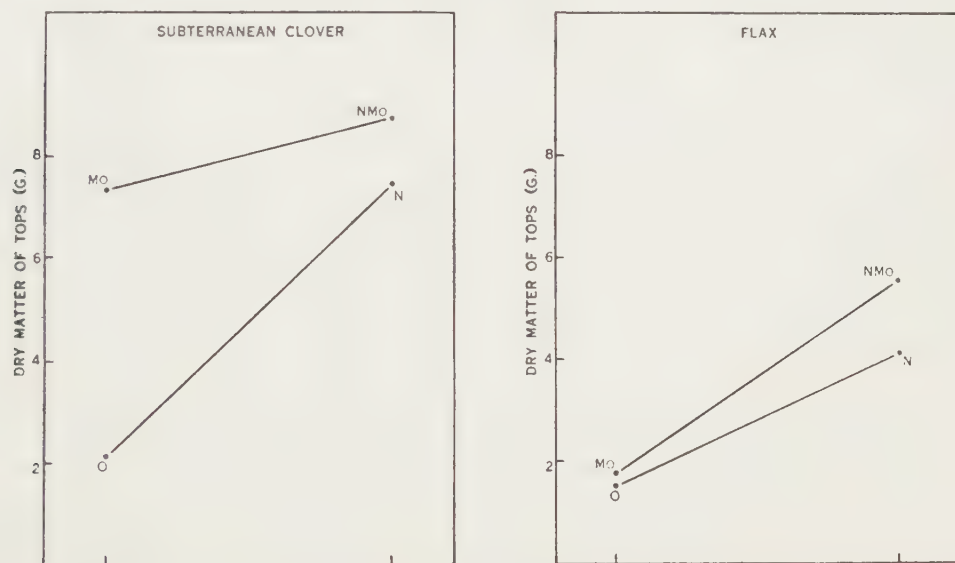


Fig. 1.—Illustrating the contrast in the interaction of nitrogen and molybdenum on a legume (subterranean clover, Experiment 12) and a non-legume (flax, Experiment 4). Soil B.

III. RESULTS

(a) Effect of Molybdenum on Symbiotic Nitrogen Fixation

Molybdenum-deficient subterranean clover was restricted in growth (Tables 1 and 2), and typically nitrogen-deficient in appearance (Plate 1). The percentage nitrogen in the molybdenum-deficient clover was invariably low (Tables 2, 7, and 8, and Fig. 2). Symbiotic nitrogen fixation was therefore inhibited by the low molybdenum supply. The application of molybdenum to the soil improved the colour and increased the percentage nitrogen and growth of these plants.

Non-legumes on the same soils responded markedly to combined nitrogen (Tables 1 and 5, Fig. 1), but the nitrogen-deficient, untreated plants did not respond to molybdenum. This indicates that the increase in the nitrogen content of the clover resulting from the application of molybdenum was due entirely to an increase in symbiotic nitrogen fixation.

TABLE 1
EFFECTS OF NITRIC ACID AND SODIUM MOLYBDATE ON YIELDS OF SUBTERRANEAN CLOVER AND ON OATS; EXPERIMENT 11
(G. dry matter per pot)

	Subterranean Clover				Oats			
	Nil	Na_2MoO_4	HNO_3	$\text{HNO}_3 + \text{Na}_2\text{MoO}_4$	Nil	Na_2MoO_4	HNO_3	$\text{HNO}_3 + \text{Na}_2\text{MoO}_4$
Soil A	14.50	17.75	20.50	19.50	2.05	1.90	12.75	13.40
Soil B	7.15	10.30	13.15	12.80	4.05	3.85	12.50	11.20
Sig. diff. for $P < 0.05$			2.82				1.81	
Sig. diff. for $P < 0.01$			3.86				2.50	

(b) Yield Interactions

Clover deficient in molybdenum responded in colour and growth to nitrogen applied either as nitric acid or as ammonium sulphate (Tables 1 and 2, Figs. 1 and 2, Plates 1 and 2). Where adequate combined nitrogen was applied the clover plants did not respond to molybdenum and in all cases the response of clover to molybdenum was greatest where no combined nitrogen was added. The interaction was therefore negative.

TABLE 2
EFFECTS OF NITRIC ACID AND SODIUM MOLYBDATE ON THE YIELD AND NITROGEN CONTENT OF SUBTERRANEAN CLOVER; EXPERIMENT 9, SOIL D TREATED WITH MANGANESE SULPHATE
(Mean transformation values in italics)

Treatments	Dry Matter per pot (g.)	Total Nitrogen (%)		Total Nitrogen (mg.)	
No N or Mo	2.8	2.00	0.299	56	1.735
Nitric acid	6.3	2.38	0.377	150	2.176
Sodium molybdate	7.4	3.21	0.506	237	2.375
Sodium molybdate + nitric acid	7.8	3.04	0.482	237	2.377
Sig. diff. for $P < 0.05$	1.7		0.079		0.263
Sig. diff. for $P < 0.01$	2.3		0.114		0.436

The nitric acid used contained less than 0.01 p.p.m. of molybdenum. The maximum amount of nitric acid added therefore supplied less than 0.01 μg . molybdenum per pot. The nitric acid treatment decreased the concentration and amount of molybdenum in the plants (Table 3), probably by its influence

TABLE 3
EFFECTS OF NITRIC ACID AND MANGANESE SULPHATE ON THE UPTAKE OF MOLYBDENUM
(Mean transformation values in italics)

	Subterranean Clover Soil D Treated with 482 µg. Mo (Experiment 9)		Subterranean Clover Soil Treated with 276 µg. Mo (Experiment 11)		Subterranean Clover Soil B (Experiment 12)		Flax Soil B Treated with 482 µg. Mo (Experiment 4)	
	Without MnSO ₄	With MnSO ₄	Soil A	Soil B	Without Mo	With 60 µg. Mo	Without MnSO ₄	With MnSO ₄
Mo per Kg. (mg.)								
No N	7.52	3.09	7.24	17.79	0.17	1.48		
	1.874	1.476	1.859	2.250	0.229	1.169		
Nitric acid	1.89	0.73	2.17	9.44	0.11	1.15	4.46	0.72
	1.274	0.865	1.336	1.975	0.039	1.061	1.647	0.857
Mo in tops (µg.)								
No N	74	23	128	183	0.38	7.38		
	1.870	1.340	3.107	3.261	0.579	1.864		
Nitric acid	21	6	42	121	0.56	6.23	15	4
	1.326	0.738	2.626	3.081	0.749	1.794	2.179	1.601
Mo per Kg. (mg.)								
Sig. diff. for $P < 0.05$	0.260		0.131		0.117		0.226	
Sig. diff. for $P < 0.01$	0.432		0.199		0.194		0.415	
Mo in tops (µg.)								
Sig. diff. for $P < 0.05$	0.321		0.199		0.158		0.394	
Sig. diff. for $P < 0.01$	0.533		0.301		0.262		0.723	

on soil pH and molybdenum availability. The pH of the soil in the pots at harvest was not significantly influenced by the nitric acid (Table 4), but increase in acidity would be expected to have occurred, particularly prior to the absorption of the nitrate by the plants.

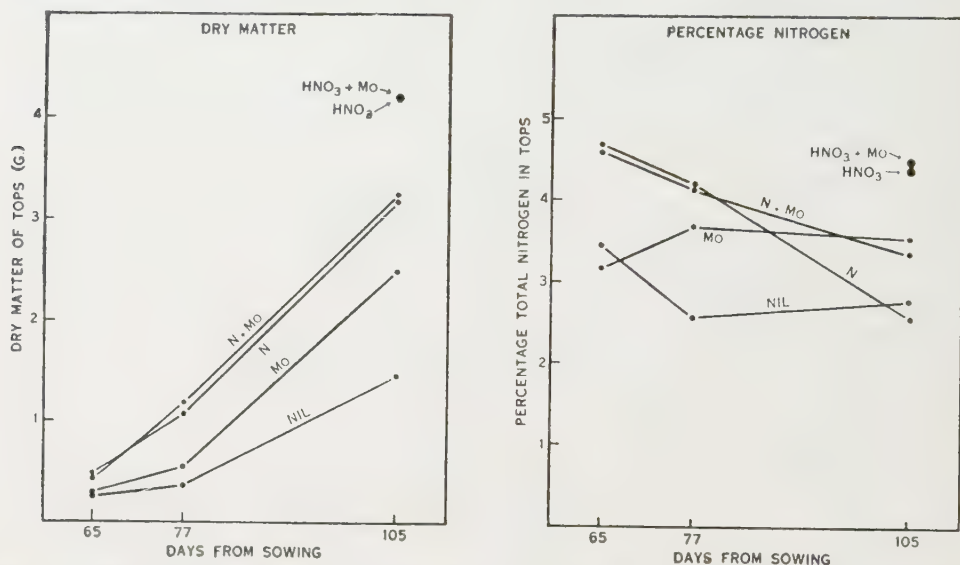


Fig. 2.—Effects and interaction of nitrogen and molybdenum on the yield and percentage nitrogen of subterranean clover at three harvests (Experiment 10). The nitrogen was applied as sulphate of ammonia in the main trial. The values obtained where nitric acid was used are also given.

The results obtained with nitric acid show that, on the soils studied, clover was deficient in molybdenum for symbiotic nitrogen fixation, but not for the

TABLE 4
EFFECTS OF MANGANESE SULPHATE AND NITRIC ACID ON MOLYBDENUM-DEFICIENT SUBTERRANEAN CLOVER; EXPERIMENT 9, SOIL D

	Nil	Nitric Acid	Manganese Sulphate	Manganese Sulphate + Nitric Acid	Manganese Sulphate + Sodium Molybdate	Manganese Sulphate + Sodium Molybdate + Nitric Acid	Sig. Diff. for $P < 0.05$	Sig. Diff. for $P < 0.01$
Dry matter (g.)	5.8	7.7	2.7	6.5	7.1	7.9	1.20	1.66
Final pH of the soil	5.32	5.34	5.21	5.28	5.21	5.27	0.31	0.43
Relative estimated weight of nodules	48	47	47	45	47	34	7	10

utilization of the added nitrate nitrogen. Even a substantial reduction in molybdenum uptake did not inhibit nitrate utilization.

Manganese sulphate reduced the uptake of molybdenum by subterranean clover (Table 3), and accentuated nitrogen deficiency (Table 4 and Plate 1, Figs. 1 and 2). It also reduced the uptake of molybdenum by flax (Table 3), and in one of the trials with soil *B* induced molybdenum deficiency in flax provided with ammonium sulphate (Table 5). In the same trial, where nitric acid was used, manganese sulphate also provided the additional sulphur needed for the normal growth of flax, and induced molybdenum deficiency (Tables 5 and 6). In the presence of manganese sulphate on this soil, flax responded to molybdenum only where nitrogen was provided. The interaction was positive and significant (Fig. 1).

TABLE 5

EFFECT OF MANGANESE SULPHATE ON THE RESPONSE OF FLAX AND OATS TO SODIUM MOLYBDATE WHEN GROWN WITHOUT NITROGEN AND WITH AMMONIUM SULPHATE AND NITRIC ACID; EXPERIMENTS 4 AND 5, SOIL *B*

Treatments	Dry Matter of Flax (g.)		Dry Matter of Oats (g.)	
	Without Manganese Sulphate	With Manganese Sulphate	Without Manganese Sulphate	With Manganese Sulphate
No Mo, S, or N	1.55	1.50	2.00	2.95
Sodium molybdate	1.45	1.75	2.05	3.00
Ammonium sulphate	4.75	3.70	9.80	9.45
Ammonium sulphate + sodium molybdate	4.85	5.00	9.50	9.75
Nitric acid	3.05	4.10	6.20	10.40
Nitric acid + sodium molybdate	3.40	5.55	5.50	10.65
Sig. diff. for $P < 0.05$		0.83		0.92
Sig. diff. for $P < 0.01$		1.17		1.29

The results with flax therefore show a striking contrast to those obtained with clover. Flax required treatment with both nitrogen and molybdenum for normal growth, while either molybdenum or nitrogen promoted normal growth of clover.

(c) *Effect of Molybdenum on the Nitrogen Fractions in Plants*

Molybdenum increased both the percentage and total amount of nitrogen in the clover plants (Tables 2, 7, and 8), and increased the percentage protein nitrogen (Table 8). The percentage of non-protein nitrogen was also increased by the molybdenum (Tables 7 and 8). There was therefore no evidence that the added molybdenum was needed by the clover for the conversion of the non-protein to protein nitrogen. The results are consistent only with the hypothesis that molybdenum increased the nitrogen supply by increasing symbiotic nitrogen fixation.

On pots treated with nitrate nitrogen (Table 8), the addition of molybdenum increased the percentage protein nitrogen in flax (main effect significant at $P < 0.01$). Molybdenum did not increase the percentage total nitrogen. The yield and total amount of protein nitrogen was increased, but the

total amount of non-protein nitrogen was unaffected by molybdenum. Tests with diphenylamine showed high nitrate accumulation in the molybdenum-deficient flax treated with manganese sulphate and nitric acid, but not in the molybdenum-treated plants. This confirmed the recorded decrease in percentage non-protein nitrogen (Table 8). In flax, therefore, molybdenum was needed for the efficient conversion of non-protein to protein nitrogen.

TABLE 6

EFFECT OF MOLYBDENUM ON THE YIELD OF FLAX, OATS, AND SUBTERRANEAN CLOVER, ON DIFFERENT SOILS TREATED WITH 5 CWT. MANGANESE SULPHATE PER ACRE (NON-LEGUMES PROVIDED ALSO WITH NITROGEN AS NITRIC ACID; EXPERIMENTS 1-9)

Species	Soil A		Soil B		Soil C		Soil D	
	Nil	Na ₂ MoO ₄	Nil	Na ₂ MoO ₄	Nil	Na ₂ MoO ₄	Nil	Na ₂ MoO ₄
Subterranean clover (g.)	7.7	14.1*	2.1	4.0*	11.9	14.8*	2.8	7.4*
Flax (g.)	6.6	6.8	4.1	5.5*	10.5	9.7		
Oats (g.)	12.3	12.7	10.4	10.6	12.8	12.9	11.2	11.4

* Increase due to molybdenum significant at $P < 0.01$.

(d) *Progressive Effects of Molybdenum and Nitrogen on Legumes*

Treatment of clover seedlings with combined nitrogen increased the percentage nitrogen and improved the colour and growth before any active symbiotic nitrogen fixation occurred. Molybdenum was without effect at this early stage (Fig. 2).

TABLE 7

EFFECTS OF SODIUM MOLYBDATE AND NITRIC ACID ON THE YIELD, PROTEIN NITROGEN, AND TOTAL NITROGEN OF SUBTERRANEAN CLOVER; EXPERIMENT 10, SOIL B, HARVEST 3

Treatments	Dry Matter (g.)	Total Nitrogen (%)	Total Nitrogen (mg.)	Protein Nitrogen (%)	Protein Nitrogen (mg.)	Non-Protein Nitrogen (%)	Non-Protein Nitrogen (mg.)	Number of Nodules
No Mo or N	1.54	2.50	38.5	1.92	29.6	0.58	8.9	364
Sodium molybdate	2.40	3.07	73.6	2.01	48.2	1.05	25.4	83
Nitric acid	4.18	3.73	154.8	2.53	105.8	1.19	49.0	1
Nitric acid + sodium molybdate	4.19	3.92	164.3	2.62	109.7	1.30	54.6	1
Sig. diff. for $P < 0.05$	0.42	0.68	14.1	0.25	9.4	0.52	16.1	
Sig. diff. for $P < 0.01$	0.58	1.03	21.4	0.38	14.2	0.78	24.4	

Later, molybdenum increased the yield and percentage nitrogen. In the case cited (Fig. 2), where ammonium sulphate was applied the percentage nitrogen fell as the plant developed. Hence, while only combined nitrogen

increased the percentage nitrogen at the first harvest, only the molybdenum increased the percentage nitrogen at the last harvest. In the same experiment, the amount of nitrogen applied as nitric acid was sufficient to maintain a high percentage nitrogen, and molybdenum had no effect on either the yield or percentage nitrogen of these nitrogen-treated plants. The results show further the dependence of symbiotic nitrogen fixation on molybdenum.

(e) Nodulation

Molybdenum decreased the number of nodules on subterranean clover, as did combined nitrogen. Abnormally large numbers of nodules occurred on plants deficient in molybdenum and nitrogen (Tables 7 and 9), indicating that under conditions of inactive symbiotic nitrogen fixation and low nitrogen supply the susceptibility of the plant to infection is greatly increased.

TABLE 8

EFFECT OF SODIUM MOLYBDATE ON YIELD, PROTEIN, AND TOTAL NITROGEN OF SUBTERRANEAN CLOVER (EXPERIMENT 1, SOIL A) AND FLAX (EXPERIMENT 4, SOIL B), TREATED WITH 5 CWT. MANGANESE SULPHATE PER ACRE (FLAX PROVIDED ALSO WITH NITROGEN AS NITRIC ACID)

	Dry Matter (g.)	Total Nitrogen (%)	Total Nitrogen (mg.)	Protein Nitrogen (%)	Protein Nitrogen (mg.)	Non-Protein Nitrogen (%)	Non-Protein Nitrogen (mg.)
Subterranean clover—							
No molybdate	7.70	1.95	150	1.61	124	0.33	26
Sodium molybdate	14.15†	2.97†	420†	2.46†	347†	0.51†	73†
Flax—							
No molybdate	4.10	2.48	102	1.43	59	1.05‡	43
Sodium molybdate	5.55†	2.48	138	1.70	95*	0.77	43

* Effect due to molybdenum significant at $P < 0.05$.

† Effect due to molybdenum significant at $P < 0.01$

‡ High nitrate in this treatment shown by diphenylamine test.

The effect of molybdenum on nodule size was markedly influenced by the sulphur status of the plant (Table 9). Under conditions of moderate sulphur deficiency, molybdenum more than doubled the yield of the clover but did not affect the number of nodules attaining a length of over 2 mm. Mean size was not determined, but may well have been increased by molybdenum as a result of the reduction in the proportion of small, late-formed nodules. Where sulphur was present, providing optimum conditions for symbiotic nitrogen fixation, molybdenum greatly increased the number of large nodules. This occurred even though molybdenum decreased the total number of nodules, indicating that molybdenum had a positive effect on nodule size.

Treatment with combined nitrogen, which depresses symbiotic nitrogen fixation, decreased the number of large nodules, particularly under conditions of sulphur deficiency. (For full data on sulphur in these studies see Anderson and Spencer 1950.)

TABLE 9
EFFECTS OF NITRIC ACID AND SODIUM MOLYBDATE AT TWO LEVELS OF SODIUM SULPHATE ON THE YIELD AND NODULATION OF SUBTERRANEAN CLOVER;
EXPERIMENT 12, SOIL B
(Mean transformation values in *italics*)

Treatments	Dry Matter (g.)	Number of Nodules per 10 cm. of Tap Root	Number of Nodules per 10 cm. of First Order Branch Roots	Number of Nodules per 10 cm. of Second Order Branch Roots	Number of Nodules per 2 mm. in Length	Relative Estimated Weight of Nodules
Without sulphate						
No Mo or N	2.20 <i>1.48</i>	1.77 <i>0.55</i>	3.43 <i>1.60</i>	2.00 <i>1.36</i>	9.7 <i>1.14</i>	63 <i>1.76</i>
Sodium molybdate	4.77 <i>2.18</i>	1.10 <i>0.42</i>	0.97 <i>1.19</i>	0.40 <i>0.83</i>	10.7 <i>1.16</i>	8 <i>0.90</i>
Nitric acid	5.00 <i>2.24</i>	0.43 <i>0.20</i>	0.50 <i>1.00</i>	0.27 <i>0.74</i>	2.0 <i>0.78</i>	4 <i>0.57</i>
Nitric acid + sodium molybdate	5.25 <i>2.29</i>	1.33 <i>0.48</i>	0.07 <i>0.83</i>	0.13 <i>0.62</i>	2.3 <i>0.80</i>	4 <i>0.57</i>
With sulphate						
No Mo or N	2.13 <i>1.46</i>	0.87 <i>0.36</i>	3.27 <i>1.58</i>	4.63 <i>1.65</i>	9.3 <i>1.06</i>	80 <i>1.90</i>
Sodium molybdate	7.27 <i>2.68</i>	1.10 <i>0.42</i>	1.13 <i>0.83</i>	0.87 <i>1.03</i>	19.0 <i>1.36</i>	30 <i>1.48</i>
Nitric acid	7.42 <i>2.72</i>	1.10 <i>0.42</i>	0.40 <i>0.99</i>	0.23 <i>0.69</i>	6.3 <i>1.00</i>	7 <i>0.80</i>
Nitric acid + sodium molybdate	8.70 <i>2.95</i>	1.10 <i>0.42</i>	0.13 <i>0.87</i>	0.10 <i>0.59</i>	14.7 <i>1.26</i>	8 <i>0.90</i>
Sig. diff. for $P < 0.05$	0.23	<i>0.20</i>	<i>0.23</i>	<i>0.29</i>	<i>0.24</i>	<i>0.28</i>
Sig. diff. for $P < 0.01$	0.30	<i>0.28</i>	<i>0.32</i>	<i>0.40</i>	<i>0.33</i>	<i>0.39</i>

IV. DISCUSSION

Molybdenum-deficient clover showed typical symptoms of nitrogen deficiency, with pale green to yellow leaves and red-brown coloration of the stems. When provided with combined nitrogen the clover did not show these symptoms.

In flax, molybdenum deficiency induced by added manganese sulphate was not sufficiently acute to produce visual symptoms. Chlorosis is known to be a symptom of acute molybdenum deficiency in non-legumes as well as legumes.

In addition, deficiency of molybdenum in plant metabolism also induces characteristic lesions in several plant species (Arnon and Stout 1939; Piper 1940; Hewitt and Jones 1947; Mulder 1948; Dunne and Jones 1948; Millikan 1948; Vanselow and Datta 1949).

Deficiency of protein results from molybdenum deficiency both in clover and flax. The correlation between leaf colour and protein content is well known (Walkley 1940; Hanson 1941). However, the comparison of effects of molybdenum on a legume and a non-legume grown in a deficient soil show that the reasons for the increase in protein content is not the same in both cases.

Yield interactions between molybdenum and combined nitrogen on legumes were negative, response to molybdenum being much greater where the plants were not provided with combined nitrogen but depended on symbiotic nitrogen fixation for their nitrogen supply. Both combined nitrogen and molybdenum increased the yield and protein content of clover by increasing the nitrogen supply—molybdenum doing so by increasing symbiotic nitrogen fixation.

Yield interactions between molybdenum and combined nitrogen on flax were positive, the need for molybdenum being greater where combined nitrogen was provided. The molybdenum was needed by flax for the utilization of the combined nitrogen. The nitrogen-molybdenum interaction on yield obtained here with flax is of the same type as the nitrogen-phosphorus interaction (Anderson and Thomas 1946) and the nitrogen-sulphur interaction (Anderson and Spencer 1949, 1950) on non-legumes and on legumes with insufficient molybdenum for optimum symbiotic nitrogen fixation.

The difference in effects of molybdenum on clover and flax is also shown by the nitrogen fractions in the plant tops. Molybdenum increased the percentage non-protein as well as the percentage protein nitrogen in the clover, thus increasing the percentage total nitrogen. In flax, molybdenum increased the percentage protein nitrogen and decreased the percentage non-protein nitrogen. The non-protein nitrogen fraction includes nitrate which accumulated in the flax provided with nitrate nitrogen but without molybdenum. By contrast, no nitrate was detected in the pale green molybdenum-deficient clover. The results show that whereas the added molybdenum was needed by the clover for symbiotic nitrogen fixation, it was needed by the non-legume for protein formation from the absorbed combined nitrogen.

The soils used, though deficient in available molybdenum for symbiotic nitrogen fixation, were not absolutely deficient in this element. The need for molybdenum in oats is established (Piper 1940), yet oats did not respond to molybdenum in any of the present trials, and presumably obtained sufficient molybdenum from the soil for their requirements. Clover did not respond at all to molybdenum in some of the experiments where nitrate nitrogen was provided, and some molybdenum was detected in these plants. It is quite probable that molybdenum is needed for metabolism in the host legume, as in non-legumes. However, the concentration of molybdenum in clover plants deficient in molybdenum for symbiotic nitrogen fixation was higher than in

the normal plants provided only with combined nitrogen. Thus the molybdenum level adequate for the utilization of nitrate nitrogen was insufficient for symbiotic nitrogen fixation.

With inadequate molybdenum and inadequate nitrogen the nodulated clover plants were stunted by nitrogen deficiency, even though nodules contain a much higher concentration of molybdenum than other plant organs (Bertrand 1940; Jensen and Betty 1943), and notwithstanding the fact that the concentration of molybdenum in the stunted plants was more than was needed for the utilization of combined nitrogen. This suggests that much higher concentrations of molybdenum are needed in the nodules for symbiotic nitrogen fixation than in the plant for the utilization of combined nitrogen.

Some of the apparent anomalies in the literature can be explained on the basis of a differential need for molybdenum by plants, depending upon the nitrogen source. In media with a sufficiently low content of available molybdenum, responses to molybdenum would probably occur irrespective of the nitrogen source. Vanselow and Datta (1949) have reported responses of citrus plants in water culture to molybdenum when provided either with ammonium or nitrate nitrogen. In media of limited purity with respect to molybdenum, whether soils or artificial culture, additional molybdenum would be needed, only for nitrogen fixation. The nature of effects of molybdenum on plants in the field would then depend upon the degree of deficiency and on the nitrogen supply.

The results obtained here with flax grown in soil provided with nitrate nitrogen are in agreement with the results obtained by Mulder (1948) with tomato plants grown in water culture. Mulder found that molybdenum increased the protein content and decreased the nitrate content of tomato plants where nitrate nitrogen was used. Hewitt, Jones, and Williams (1949) found that deficiency of molybdenum in cauliflower plants resulted in a considerable reduction in the concentration of most amino acids in the plant. Accumulation of nitrate in molybdenum-deficient plants has frequently been recorded (Hewitt and Jones 1947; Wilson and Waring 1948; Mulder 1948; Stout and Meagher 1948).

Strict comparison of the need for molybdenum in the plant for the utilization of nitrate and ammonium nitrogen is not possible in the present experiments where the nitrogen compounds were applied to the soil, as nitrification of the ammonia might be expected. However, it is interesting to note that comparable responses were obtained with flax where either nitric acid or ammonium sulphate was used, and that no nitrate accumulation was detected with diphenylamine in the molybdenum-deficient flax where ammonium sulphate was used. Mulder (1948) obtained evidence suggesting that plants provided with ammonium nitrogen do not require molybdenum, although Vanselow and Datta (1949) obtained similar response of citrus plants to molybdenum in water cultures supplying either ammonium or nitrate nitrogen.

Increase in symbiotic nitrogen fixation with molybdenum was associated with a decrease in the number of nodules. However, plants provided with

molybdenum were sufficiently well nodulated to provide for their nitrogen requirements, suggesting that reduction in nodulation was not due to a direct inhibiting effect of molybdenum on *Rhizobium*, but was brought about as a result of the increase in nitrogen fixation. Combined nitrogen also frequently reduces nodulation. It checks the deformation of the root hairs by *Rhizobium*, thus preventing infection, and checks the growth of nodules already formed (Thornton 1935). The mechanism by which these effects are brought about is not understood, but both carbohydrate and nitrogen in the plant may be involved (Wilson 1940; Thornton 1947). It is interesting to note that, in the present experiments, the decrease in the number of nodules formed where molybdenum was applied was associated with an increase in the percentage of both non-protein and protein nitrogen.

There is strong additional evidence that decreased nodulation with molybdenum was not due to a direct inhibiting effect of molybdenum on *Rhizobium*. The greatest decrease in the number of nodules on lucerne (Anderson and Oertel 1946) occurred with the two lowest levels of molybdenum, and little further decrease resulted from a more than 200-fold increase in concentration. In the absence of lime, where the plants were too poorly nodulated for their nitrogen requirements, molybdenum caused no decrease at all in the number of nodules.

In the present experiments molybdenum decreased the number of smaller nodules on the later-formed root branches. This decrease in the number of smaller nodules would have tended to increase the mean size of nodules. However, there was evidence that molybdenum had a positive effect on nodule size, as the molybdenum-treated plants had more nodules over 2 mm. in length than did the molybdenum-deficient plants with their greater number of nodules. This occurred particularly where sulphur was adequate for optimum nitrogen fixation, and might be expected to result from the increase in nitrogen fixation in the nodules.

Burk (1934) reported responses of *Azotobacter* to either molybdenum or combined nitrogen, and absence of response to molybdenum where combined nitrogen was provided. This result indicated that molybdenum is particularly needed by *Azotobacter* for nitrogen fixation. Bortels (1936) and others later obtained responses of *Azotobacter* to molybdenum where nitrate nitrogen, asparagine, or ammonium nitrogen was used. The results of Burema and Wieringa (1942) and Mulder (1948), however, have established the particular need of *Azotobacter* for molybdenum when dependent on elemental nitrogen.

The complete absence of response of *Azotobacter* to molybdenum in the early experiments where combined nitrogen was provided was not necessarily due to any impurity of molybdenum in the nitrogen compounds used. It can be explained on the basis of the differential need for molybdenum depending upon the nitrogen source, molybdenum impurity in the basal medium being sufficient for the utilization of combined nitrogen, but not for nitrogen fixation.

The results here obtained with legumes are therefore in agreement with those obtained by others with *Azotobacter*. They indicate that molybdenum

is needed by legumes in the symbiotic nitrogen fixation process, either in the initial reaction with gaseous nitrogen or in a later reaction which is not an essential step in either nitrate or ammonium utilization.

V. ACKNOWLEDGMENTS

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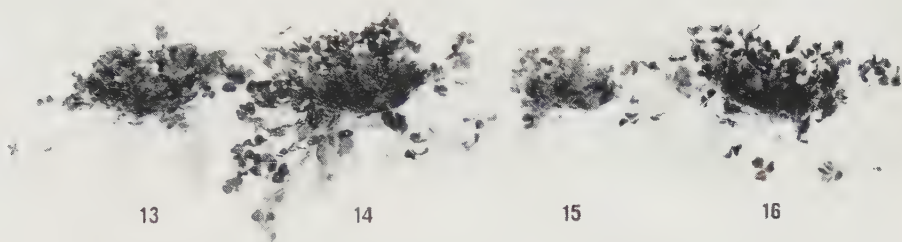


Fig. 1.—Molybdenum deficiency in subterranean clover accentuated by manganese sulphate (Experiment 9).

- (13) No molybdenum.
- (14) 1 lb. Sodium molybdate per acre.
- (15) 5 cwt. Manganese sulphate per acre.
- (16) 1 lb. Sodium molybdate + 5 cwt. manganese sulphate per acre.

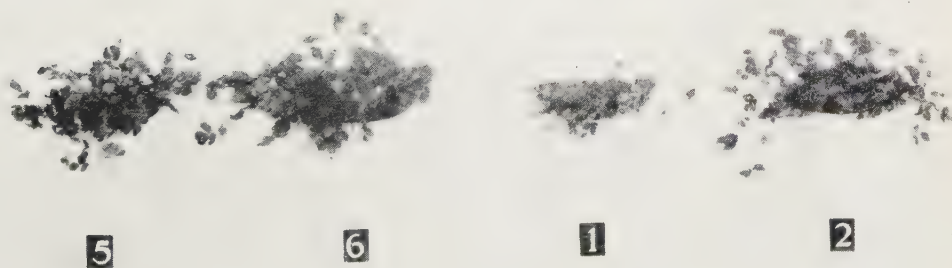


Fig. 2.—Nitrogen deficiency in subterranean clover accentuated by decreasing molybdenum uptake with manganese sulphate (Experiment 9).

- (5) No molybdenum.
- (6) Nitric acid (100 mg. N per pot).
- (1) 5 cwt. Manganese sulphate per acre.
- (2) Nitric acid (100 mg. N per pot) + 5 cwt. manganese sulphate per acre.

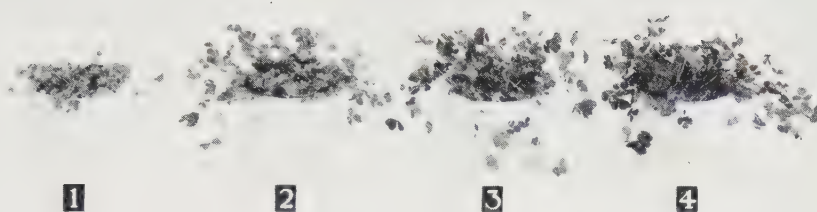


Fig. 1.—Molybdenum-nitrogen interaction on subterranean clover in the presence of manganese sulphate (Experiment 9).

- (1) No molybdenum.
- (2) Nitric acid (100 mg. N per pot).
- (3) 1 lb. Sodium molybdate per acre.
- (4) Nitric acid (100 mg. N per pot) + 1 lb. sodium molybdate per acre.



Fig. 2.—Molybdenum-nitrogen interaction on subterranean clover without manganese sulphate on a soil more deficient in molybdenum (Experiment 12).

- (21) No molybdenum.
- (22) 2 oz. Sodium molybdate per acre.
- (23) Nitric acid (115 mg. N per pot).
- (24) 2 oz. Sodium molybdate per acre + nitric acid (115 mg. N per pot).

SULPHUR IN NITROGEN METABOLISM OF LEGUMES AND NON-LEGUMES

By A. J. ANDERSON* and D. SPENCER*

[Manuscript received June 7, 1950]

Summary

A study was made of the influence of sulphur on the nitrogen metabolism of subterranean clover (*Trifolium subterraneum* L.) and the non-legumes oats (*Avena sativa* L.), flax and linseed (*Linum usitatissimum* L.), and *Paspalum dilatatum* Poir. All these species responded to fertilizers containing sulphur on the three soils used. Sulphates of ammonium, potassium, sodium, and manganese were all effective.

Non-legumes deficient in either sulphur or nitrogen were pale green and contained a lower percentage of protein nitrogen than the corresponding dark green treated plants. Further, nitrogen deficiency decreased the percentages of non-protein nitrogen and total nitrogen. Sulphur deficiency increased the percentages of both non-protein and total nitrogen. High nitrate accumulation occurred in the sulphur-deficient plants where nitrate was the source of nitrogen. Thus sulphur was needed by the non-legumes for protein formation from the absorbed combined nitrogen. Nitrogen-deficient non-legumes responded little in colour or growth to sulphur. The sulphur-nitrogen yield interaction was positive.

Clover, under molybdenum supply inadequate for symbiotic nitrogen fixation, reacted to sulphur and nitrogen treatments in the same way as the non-legumes, normal growth occurring only where both sulphur and nitrogen were provided. No effect of sulphur on the yield or visual symptoms was obtained where molybdenum and nitrogen were deficient. The molybdenum-sulphur and nitrogen-sulphur yield interactions were positive. The added sulphur and nitrogen were needed for protein formation in the legume as in the non-legumes. In clover provided with molybdenum and dependent upon symbiotic nitrogen fixation for its nitrogen supply, deficiency of sulphur decreased the percentage total nitrogen as well as the yield. Thus symbiotic nitrogen fixation was greatly reduced by sulphur deficiency.

Clover deficient in sulphur showed visual symptoms of nitrogen deficiency, and contained a low percentage of protein nitrogen. However, the percentage of non-protein nitrogen in the clover was increased by sulphur deficiency. The sulphur-deficient clover responded little in colour or growth to applied nitrogen. Thus the restricted growth of the sulphur-deficient clover was not due to poor nitrogen fixation consequent on defective nodulation, but to a deficiency of sulphur in the host legume.

Clover deficient in sulphur had fewer nodules than the treated plants. Nodulation reflected the nitrogen demand of the host legume. Sulphur deficiency decreased the demand for nitrogen and decreased the number of nodules, in contrast to deficiencies of nitrogen and molybdenum, which increased the demand for nitrogen and the number of nodules.

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I. INTRODUCTION

It has long been known that, in regions where sulphur-deficient soils occur, legumes are particularly responsive to sulphur-containing fertilizers (Hart and Tottingham 1915; Conrad 1941). It has frequently been reported that elementary sulphur and sulphates increase the percentage nitrogen as well as the yield of legumes on such deficient soils; and it has been suggested that sulphur indirectly affects legumes through its direct effect upon the nitrogen-fixing organisms (Neller 1926).

Sulphur is contained in the amino acids cystine and methionine, which occur in plant proteins. The role of sulphur in plants, particularly in protein formation, has been reviewed by Wood (1942). The present experiments were carried out to compare the effects of sulphur on the nitrogen metabolism of legumes and non-legumes.

II. EXPERIMENTAL

(a) General

Plants were grown on sulphur-deficient soils in pots in an unheated glass-house. The 'Tallarook' strain of subterranean clover was used in Experiments 7 and 8, which were sown in late spring, and the Bacchus Marsh strain in the other trials. Algerian oats (*Avena sativa* L.), *Paspalum dilatatum* Poir., and the flax and linseed varieties of *Linum usitatissimum* L. were the non-legumes used. The clover seed was inoculated with an effective strain of *Rhizobium* before sowing.

Ten plants were grown in each glazed earthenware pot used in all trials except Experiment 6, and five plants were grown in each glass pot in Experiment 6. All plants were harvested shortly before flowering or when the first few flowers had formed. Immediately after harvest, the plant tops were dried rapidly under forced draught at 50°C. for yield determination and chemical analyses.

Unless otherwise stated, nutrients other than nitrogen were applied in solution at the beginning of each experiment. Nitrogen fertilizers were applied in small doses at intervals, the nitric acid in solutions containing up to 0.2 per cent. nitric acid. The levels of the dressings applied are expressed as cwt. per acre based on the surface area of the pot, and the equivalents in mg. per pot are given for each treatment. Randomization of treatments was adopted throughout.

Weights of oven-dry soil in each pot are given in the details of particular experiments. The pots were weighed frequently and the soils watered to field capacity. The soils used were all from the Southern Tablelands of New South Wales. These are identified in the text as:

Soil A.—a grey-brown loam from the Dickson Experiment Station, C.S.I.R.O., Canberra, A.C.T.

Soil B.—a light, grey-brown sandy loam of sedimentary origin, from Kingsdale, near Goulburn, N.S.W.

Soil C.—a brown to reddish brown loam of basaltic origin from Roslyn, N.S.W.

The transformation values used in the statistical analysis of the data to secure uniformity of variance within treatments were the square root transformation for dry matter (Table 12), log transformations of the form $A + \log(x + B)$ for the data on nodulation, and log transformations of the form $A + \log x$ for the data on chemical analysis. The constant A has been introduced into these transformations primarily to avoid negative values.

(b) Details of Experiments

Experiment 1.—To test the effects of manganese sulphate and ammonium sulphate on subterranean clover, at different levels of application of molybdenum. *Soil A.* Sown June 3, 1948 and harvested October 7. A $2 \times 2 \times 2$ factorial combination of the following fertilizers was used:

Manganese sulphate — nil or 5 cwt. per acre ($\equiv 680$ mg. per pot).

Ammonium sulphate — nil or 7 cwt. per acre ($\equiv 952$ mg. per pot).

Sodium molybdate — nil or 1 lb. per acre ($\equiv 1.21$ mg. per pot).

Replications: 2. *Soil:* 1.51 Kg. of soil *A* in glazed earthenware pots, with 3 cwt. of monocalcium phosphate per acre on all pots.

Experiment 2.—Details as for Experiment 1. *Soil B.* (1.48 Kg. of soil per pot — harvested September 21.)

Experiment 3.—Details as for Experiment 1. *Soil C.* (1.22 Kg. of soil per pot.)

Experiment 4.—To test the effect of manganese sulphate on flax, at different levels of application of nitric acid, ammonium sulphate, and molybdenum. *Soil B.* Sown June 3, 1948 and harvested October 21. A $2 \times 3 \times 2$ factorial combination of the following fertilizers was used:

Manganese sulphate — nil or 5 cwt. per acre ($\equiv 680$ mg. per pot).

Three nitrogen treatments — nil, 6.25 cwt. of ammonium sulphate per acre ($\equiv 180$ mg. N per pot), or 18 mg. N per pot applied as nitric acid.

Sodium molybdate — nil, or 1 lb. per acre ($\equiv 1.21$ mg. per pot).

Replications: 2. *Soil:* 1.47 Kg. of soil *B* in glazed earthenware pots, with 2 cwt. of monocalcium phosphate per acre on all pots.

Experiment 5.—Details as for Experiment 4, with oats as the test plant.

Experiment 6.—To test the effects of manganese sulphate and a late dressing of potassium sulphate at different levels of molybdenum on oats on soil *A* and on flax on soil *C.* Sown June 3, 1948 and harvested October 21. A $2 \times 2 \times 2$ factorial combination of the following fertilizers was used:

Manganese sulphate — nil or 5 cwt. per acre ($\equiv 680$ mg. per pot).

Potassium sulphate — nil or 1 cwt. per acre ($\equiv 136$ mg. per pot) applied twenty days before harvest.

Sodium molybdate — nil or 1 lb. per acre ($\equiv 1.21$ mg. per pot).

Replications: 1. *Soil:* 1.51 Kg. of soil *A*, or 1.22 Kg. of soil *C*, in glazed earthenware pots, with 2 cwt. of monocalcium phosphate per acre + 180 mg. of N per pot applied as nitric acid.

Experiment 7.—Details as for Experiment 6, but with flax on soil A and oats on soil C, and using 1 cwt. of manganese chloride per acre instead of the potassium sulphate treatment.

Experiment 8.—To test the effect of a late dressing of sodium sulphate on subterranean clover, at different levels of application of molybdenum. Soil B. Sown June 29, 1948 and harvested November 15. A 2×2 factorial combination of the following fertilizers was used:

Sodium sulphate—nil or 1 cwt. per acre ($\equiv 67$ mg. per pot) applied October 14.

Sodium molybdate—nil or 2 oz. per acre ($\equiv 75$ μ g. per pot).

Replications: 2. Soil: 0.80 Kg. of soil B in glass pots, with 2 cwt. of monocalcium phosphate per acre on all pots.

Experiment 9.—To test the effects of a number of sulphates and chlorides, alone and in combination, on subterranean clover. Soil A. Sown October 25, 1948 and harvested March 25, 1949. The nine fertilizer treatments were:

- (1) Nil.
- (2) Manganese sulphate at 3 cwt. per acre ($\equiv 100$ mg. Mn and 58 mg. S per pot).
- (3) Potassium sulphate ($\equiv 141$ mg. K and 58 mg. S per pot).
- (4) Manganese sulphate and potassium sulphate ($\equiv 2 + 3$ above).
- (5) Manganese chloride ($\equiv 100$ mg. Mn per pot).
- (6) Manganese chloride + potassium sulphate ($\equiv 3 + 5$ above).
- (7) Potassium chloride ($\equiv 141$ mg. K per pot).
- (8) Ferrous sulphate ($\equiv 58$ mg. S per pot).
- (9) Ferrous sulphate + manganese sulphate ($\equiv 2 + 8$ above).

Replications: 2. Soil: 1.38 Kg. of soil A in glazed earthenware pots, with 3 cwt. of monocalcium phosphate + 1 lb. of sodium molybdate per acre on all pots.

Experiment 10.—To test the effects of manganese sulphate and a number of forms of nitrogen on subterranean clover. Soil A. Sown October 25, 1948 and harvested March 24, 1949. The six nitrogen treatments were:

- (1) Nil.
- (2) Sodium nitrate.
- (3) Ammonium chloride.
- (4) Ammonium sulphate.
- (5) Urea.
- (6) Nitric acid.

Treatments 2-5 each provided 289 mg. N per pot, and the nitric acid provided 332 mg. N per pot. One set of pots with the above nitrogen treatments received 3 cwt. of manganese sulphate per acre ($\equiv 408$ mg. per pot), and the other set no manganese sulphate.

Replications: 2. Soil: 1.38 Kg. of soil A in glazed earthenware pots, with 3 cwt. of monocalcium phosphate + 1 lb. of sodium molybdate per acre on all pots.

Experiment 11.—To test the effects of manganese sulphate and potassium sulphate at different levels of nitric acid and ammonium chloride on linseed (*Linum usitatissimum* L.) and *Paspalum dilatatum* Poir. Soil C. Sown January 21, 1949 and harvested June 2. A 3×3 factorial combination of the following fertilizers was used:

Sulphate dressings — nil, $1\frac{1}{2}$ cwt. of manganese sulphate per acre ($\equiv 29$ mg. S per pot), or potassium sulphate ($\equiv 29$ mg. S per pot).

Nitrogen dressings — nil, 5 cwt. of ammonium chloride per acre ($\equiv 178$ mg. N per pot), or nitric acid ($\equiv 246$ mg. N per pot).

Replications: 2. Soil: 1.29 Kg. of soil C in glazed earthenware pots, with 1 cwt. of monocalcium phosphate + 1 lb. of sodium molybdate per acre on all pots.

Experiment 12.—To test the effect of sodium sulphate on subterranean clover, at different levels of application of nitric acid and sodium molybdate. Soil B. Sown June 14, 1949 and harvested October 13. A $2 \times 2 \times 2$ factorial combination of the following fertilizers was used:

Sodium sulphate — nil or 1 cwt. per acre ($\equiv 136$ mg. per pot).

Nitric acid — nil or dressings of 14.4 mg. N per pot on July 13, 22, and 28, August 5 and 11, and September 1, 8, and 15, then 28.8 mg. N per pot on September 22 and 29.

Sodium molybdate — nil or 2 oz. per acre ($\equiv 152$ μ g. per pot).

Replications: 4. Soil: 1.64 Kg. of soil B in glazed earthenware pots, with 2 cwt. of monocalcium phosphate + 2 cwt. of potassium chloride per acre on all pots.

Experiment 13.—To test the effect of sodium sulphate on subterranean clover, at different levels of application of ammonium nitrate and sodium molybdate. Soil A. Sown July 11, 1949 and harvested October 13. A $2 \times 2 \times 2$ factorial combination of the following fertilizers was used:

Sodium sulphate — nil or 1 cwt. per acre ($\equiv 136$ mg. per pot).

Ammonium nitrate — nil or dressings of 1 cwt. per acre ($\equiv 136$ mg. per pot) August 16 and September 2.

Sodium molybdate — nil or 2 oz. per acre ($\equiv 152$ μ g. per pot).

Replications: 3. Soil: 1.24 Kg. of soil A in glazed earthenware pots, with 2 cwt. of monocalcium phosphate + 2 cwt. of potassium chloride per acre on all pots.

(c) Analytical Methods

Plant tops, which were dried rapidly at 50°C . immediately after harvest, were then finely ground, further dried at 101°C ., and cooled in a desiccator prior to chemical analysis.

Total nitrogen was determined on 0.2 g. samples by the Kjeldahl procedure, using selenium as a catalyst. The final distillation step was carried out in a Parnas-Wagner steam distillation unit using 2 per cent. boric acid solution to trap the ammonia as described by Ma and Zuazaga (1942).

Protein nitrogen was rendered insoluble by treatment with boiling alcohol, and non-protein nitrogen constituents were extracted in alcohol and in water,

as described in an earlier paper (Anderson and Spencer 1950). Nitrogen in the plant residue was determined as described for total nitrogen.

Non-protein nitrogen was calculated by difference between the total nitrogen and protein nitrogen fractions.

Total sulphur estimations were made according to the method of Lugg and Weller (1944) involving a sodium peroxide fusion of plant material followed by precipitation of the sulphate as barium sulphate.

III. RESULTS

The soils used in the present experiments were also used in a parallel study on the role of molybdenum in nitrogen metabolism (Anderson and Spencer 1950). All the soils were shown to be deficient in available molybdenum for symbiotic nitrogen fixation in subterranean clover.

TABLE 1
EFFECTS OF AMMONIUM SULPHATE AND MANGANESE SULPHATE AT TWO LEVELS OF
MOLYBDENUM ON SUBTERRANEAN CLOVER
(Mean transformation values in italics)

Treatments	Experiment 1, Soil A					Expt. 2 Soil B	Expt. 3 Soil C
	Dry Matter (g.)	Total Nitrogen (%)		Total Nitrogen (mg.)		Dry Matter (g.)	Dry Matter (g.)
No S or Mo	7.6	1.87	0.273	143	2.156	2.5	5.7
Ammonium sulphate	11.2	1.94	0.287	217	2.337	5.1	13.8
Manganese sulphate	7.7	1.95	0.289	150	2.178	2.1	11.9
Manganese sulphate + ammonium sulphate	10.1	2.06	0.314	209	2.319	4.5	13.4
Sodium molybdate	8.5	1.93	0.286	165	2.218	2.6	5.2
Sodium molybdate + ammonium sulphate	17.6	2.57	0.411	454	2.659	5.7	17.3
Sodium molybdate + manganese sulphate	14.1	2.97	0.473	420	2.622	4.0	14.8
Sodium molybdate + manga- nese sulphate + ammonium sulphate	14.6	2.71	0.433	397	2.599	5.3	16.0
Sig. diff. for $P < 0.05$	1.4		0.040		0.056	0.9	1.35
Sig. diff. for $P < 0.01$	2.1		0.058		0.082	1.2	2.00

In the present study the influence of sulphur has been examined at different levels of molybdenum supply. Levels of molybdenum have been varied to control the nitrogen supply to the host legume, providing a means of examining more fully the nitrogen-sulphur interactions in these plants.

(a) Responses to Forms of Sulphur

Responses of plants to sulphates of potassium, ammonia, manganese, and iron, and interactions between the forms of sulphur, show the need for added

sulphur for maximum growth on all the soils used (Tables 1, 2, and 3; Plates 1-4). Sulphur-deficient subterranean clover (Table 2) contained only 0.058 per cent. sulphur, while plants provided with sulphates of manganese and potassium contained 0.319 and 0.244 per cent. sulphur respectively.

TABLE 2

EFFECTS OF SULPHATES AND CHLORIDES OF MANGANESE, POTASSIUM, AND IRON, ALONE AND IN COMBINATION, ON THE YIELD, NITROGEN CONTENT, AND SULPHUR CONTENT OF SUBTERRANEAN CLOVER; EXPERIMENT 9, SOIL A, WITH MOLYBDENUM

(Mean transformation values in *italics*)

	Dry Matter (g.)	Total Nitrogen (%)	Total Nitrogen (mg.)	Total Sulphur (%)	Total Sulphur (mg.)
No S or Cl	9.8	1.71	168 2.225	0.058	5.7 <i>0.749</i>
Manganese chloride	10.1	1.72	173 2.239	0.051	5.2 <i>0.707</i>
Manganese sulphate	15.6	2.18	339 2.530	0.319	49.8 <i>1.698</i>
Potassium sulphate	16.3	2.26	368 2.565	0.244	39.8 <i>1.593</i>
Manganese sulphate + potassium sulphate	15.5	2.34	363 2.559		
Manganese chloride + potassium sulphate	14.4				
Potassium chloride	10.3				
Ferrous sulphate	13.4				
Ferrous sulphate + manganese sulphate	13.5				
Sig. diff. for $P < 0.05$	2.0	0.20	0.079	0.077	0.156
Sig. diff. for $P < 0.01$	2.7	0.33	0.132	0.142	0.287

(b) Effects of Sulphur on Symbiotic Nitrogen Fixation

Sulphur-deficient clover not provided with combined nitrogen was pale green to yellow (Plate 1) and had a low percentage nitrogen (Tables 1 and 2). Symbiotic nitrogen fixation was therefore seriously inhibited by sulphur deficiency. In appearance the sulphur-deficient clover plants were indistinguishable from those provided with sulphur but deficient in molybdenum for symbiotic nitrogen fixation. Where molybdenum was provided, treatment with sulphates markedly increased the growth and percentage nitrogen (Tables 1 and 2) and also improved the colour and growth of the clover (see also Tables 7 and 11; Plate 2).

Nitrogen-deficient non-legumes responded much less to sulphur than did plants provided with combined nitrogen (Tables 3 and 5; Fig. 1). In some of the trials the non-legumes did not respond at all to sulphur where no combined nitrogen was provided, indicating that the increase in the nitrogen content of the legume was entirely due to an increase in symbiotic nitrogen fixation.

(c) Sulphur-Nitrogen Yield Interactions of Legumes

Treatment with combined nitrogen had little or no effect on the yield of sulphur-deficient clover (Tables 4 and 12; Fig. 1). In no case was the response to sulphur greater in the absence than in the presence of added com-

TABLE 3

EFFECTS OF NITROGEN APPLIED AS NITRIC ACID AND AMMONIUM CHLORIDE, AND OF SULPHUR APPLIED AS MANGANESE SULPHATE AND POTASSIUM SULPHATE, ALONE AND IN COMBINATION, ON THE YIELDS OF LINSEED AND PASPALUM; EXPERIMENT 11, SOIL C, WITH MOLYBDENUM

Treatment	Linseed Dry Matter (g.)			Paspalum Dry Matter (g.)		
	No N	HNO ₃	NH ₄ Cl	No N	HNO ₃	NH ₄ Cl
No S	9.3	9.2	7.0	6.1	7.0	6.1
Manganese sulphate	10.9	14.1	14.4	6.5	12.0	12.4
Potassium sulphate	11.0	13.2	15.2	7.7	11.5	12.4
Sig. diff. for $P < 0.05$		1.8			1.6	
Sig. diff. for $P < 0.01$		2.6			2.3	

bined nitrogen (Fig. 1; Plate 1, Fig. 3). Nitrogen-treated plants deficient in sulphur remained pale green, and typically nitrogen-deficient in appearance. These results contrast with effects of nitrogen on clover deficient only in molybdenum for symbiotic nitrogen fixation.

TABLE 4

EFFECTS OF VARIOUS NITROGEN TREATMENTS AND OF SULPHUR APPLIED AS MANGANESE SULPHATE ON THE YIELD AND NODULATION OF SUBTERRANEAN CLOVER AND ON THE pH OF THE SOIL; EXPERIMENT 10, SOIL A, WITH MOLYBDENUM

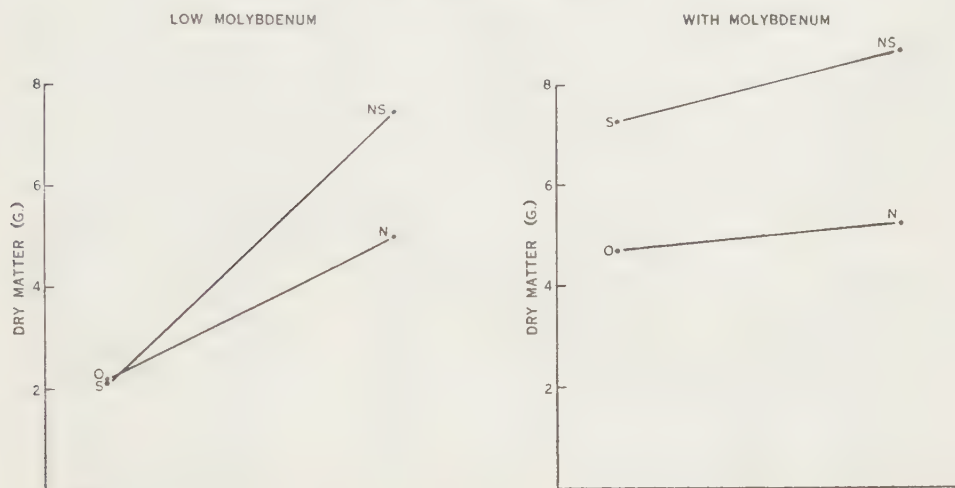
	Dry Matter per Pot (g.)		pH of Soils at Harvest		Relative Estimated Weight of Nodules	
	No S	MnSO ₄	No S	MnSO ₄	No S	MnSO ₄
No N	11.1	14.9	5.27	5.18	105	160
Sodium nitrate	11.9	15.3	5.98	5.92	85	110
Nitric acid	10.8	16.2	5.49	4.95	62	147
Ammonium chloride	9.0	14.9	4.48	4.47	37	157
Ammonium sulphate	15.8	14.1	4.48	4.34	170	135
Urea	10.5	15.4	5.41	4.77	58	162
Sig. diff. for $P < 0.05$		1.4		0.25		36
Sig. diff. for $P < 0.01$		1.9		0.34		49

Results suggest that the restricted growth of clover at low sulphur levels was not due to ineffective symbiotic nitrogen fixation, but to a deficiency of sulphur in the host legume, and that this latter condition exerted some controlling influence on symbiotic nitrogen fixation.

(d) *Yield Interactions of Non-Legumes and of Legumes Deficient in Molybdenum*

Non-legumes without added sulphur or without added nitrogen were equally pale green, in each case giving the appearance of nitrogen deficiency

SUBTERRANEAN CLOVER



FLAX

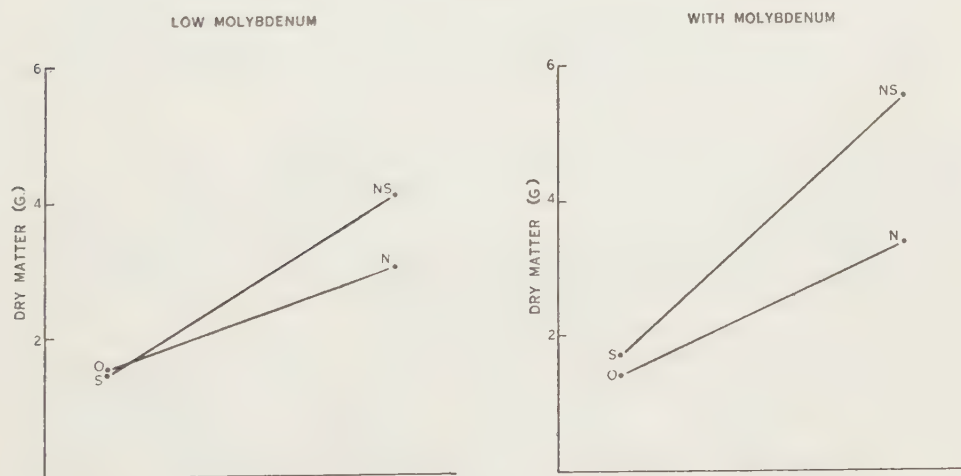


Fig. 1.—Effects and interaction of nitrogen and sulphur on a legume (subterranean clover, Experiment 12) and a non-legume (flax, Experiment 4), treated with molybdenum and untreated. Soil B.

(Plates 3 and 4). The plants grew normally and were a healthy dark green colour only where both sulphur and nitrogen were provided (Tables 3 and 5; Fig. 1; Plate 3, Fig. 2).

Clover deficient in molybdenum, though nodulated, responded to sulphur and nitrogen in the same way as did the grasses. Molybdenum-deficient clover, deficient also in sulphur and nitrogen, was pale green and of a typically nitrogen-deficient appearance (Plate 2). These plants responded little to sulphur or nitrogen applied alone, but grew normally when adequate amounts of both were provided (Tables 1 and 12; Fig. 1).

TABLE 5

EFFECTS OF MANGANESE SULPHATE, AMMONIUM SULPHATE, AND NITRIC ACID AT TWO LEVELS OF MOLYBDENUM ON THE YIELDS OF FLAX AND OATS: EXPERIMENTS 4 AND 5, SOIL B

Treatments	Dry Matter of Flax (g.)		Dry Matter of Oats (g.)	
	Without Molybdenum	With Molybdenum	Without Molybdenum	With Molybdenum
No S or N	1.55	1.45	2.00	2.05
Manganese sulphate	1.50	1.75	2.95	3.00
Ammonium sulphate	4.75	4.85	9.80	9.50
Manganese sulphate + ammonium sulphate	3.70	5.00	9.45	9.75
Nitric acid	3.05	3.40	6.20	5.50
Manganese sulphate + nitric acid	4.10	5.55	10.40	10.65
Sig. diff. for $P < 0.05$		0.83		0.92
Sig. diff. for $P < 0.01$		1.17		1.29

The results show that the added sulphur and nitrogen were equally needed in plant metabolism by the non-legumes and by the molybdenum-deficient legume.

(e) *The Nitrogen Status of the Plants*

Sulphur-deficient clover not provided with combined nitrogen contained a low percentage nitrogen, irrespective of the molybdenum supply (Tables 6 and 11). Where molybdenum was provided, sulphur markedly increased the percentage and total amount of nitrogen in the clover plants. Sulphur also increased the percentage protein nitrogen in the clover, but decreased the percentage non-protein nitrogen (Tables 6 and 7).

The percentage nitrogen of clover deficient in molybdenum was almost equally low whether sulphur was provided or not (Table 6). However, the sulphur caused more protein to be formed in these plants at the expense of non-protein nitrogen, even though it had little or no effect on growth.

Clover deficient in sulphur but provided with combined nitrogen contained a high percentage nitrogen, but the plants were pale green and as unthrifty as plants without added nitrogen (Tables 4 and 7; Plate 1, Fig. 3). Sulphur had little effect on the percentage total nitrogen in these nitrogen-treated plants, but markedly increased the percentage protein nitrogen and decreased the percentage non-protein nitrogen (Table 7). The clover provided with sulphur and nitrogen grew normally and was a healthy dark green colour.

Sulphur-deficient non-legumes not provided with combined nitrogen also contained a low percentage nitrogen. Sulphur did not increase the percentage

TABLE 6

EFFECTS OF SULPHUR AT TWO LEVELS OF MOLYBDENUM ON THE YIELD, PROTEIN, AND TOTAL NITROGEN CONTENT, AND NODULATION OF SUBTERRANEAN CLOVER; EXPERIMENT 1, SOIL A

(Mean transformation values in italics)

	Dry Matter per pot (g.)	Total Nitrogen (%)	Total Nitrogen (mg.)	Protein Nitrogen (%)	Protein Nitrogen (mg.)	Non-protein Nitrogen (%)	Non-protein Nitrogen (mg.)	Relative Estimated Weight of Nodules
No Mn or Mo	7.6	1.87 <i>0.273</i>	143 <i>2.156</i>	1.10 <i>0.043</i>	84 <i>1.926</i>	0.77 <i>0.886</i>	59 <i>1.770</i>	37
Manganese sulphate	7.7	1.95 <i>0.289</i>	150 <i>2.175</i>	1.61 <i>0.207</i>	124 <i>2.094</i>	0.33 <i>0.524</i>	26 <i>1.414</i>	49
Sodium molybdate	8.5	1.93 <i>0.286</i>	165 <i>2.218</i>	1.05 <i>0.021</i>	90 <i>1.954</i>	0.88 <i>0.946</i>	75 <i>1.881</i>	14
Sodium molybdate + manganese sulphate	14.1	2.97 <i>0.473</i>	420 <i>2.622</i>	2.46 <i>0.391</i>	347 <i>2.540</i>	0.51 <i>0.711</i>	73 <i>1.863</i>	26
Sig. diff. for $P < 0.05$	1.5	<i>0.040</i>	<i>0.056</i>	<i>0.068</i>	<i>0.083</i>	<i>0.048</i>	<i>0.108</i>	11
Sig. diff. for $P < 0.01$	2.2	<i>0.058</i>	<i>0.082</i>	<i>0.113</i>	<i>0.138</i>	<i>0.079</i>	<i>0.178</i>	14

TABLE 7

EFFECTS OF NITROGEN AND SULPHUR, ALONE AND IN COMBINATION, ON THE YIELD, PROTEIN, AND TOTAL NITROGEN CONTENT OF SUBTERRANEAN CLOVER; EXPERIMENT 10, SOIL A, WITH MOLYBDENUM

(Mean transformation values in italics)

	Dry Matter per pot (g.)	Total Nitrogen (%)	Total Nitrogen (mg.)	Protein Nitrogen (%)	Protein Nitrogen (mg.)	Non-protein Nitrogen (%)	Non-protein Nitrogen (mg.)	Relative Estimated Weight of Nodules
No N or S	10.9	1.69 <i>0.227</i>	185 <i>2.267</i>	1.07 <i>0.031</i>	117 <i>2.069</i>	0.61 <i>0.788</i>	68 <i>1.826</i>	140
Sodium nitrate	11.5	2.12 <i>0.327</i>	245 <i>2.389</i>	1.19 <i>0.076</i>	137 <i>2.138</i>	0.93 <i>0.968</i>	108 <i>2.031</i>	110
Manganese sulphate	15.8	2.10 <i>0.323</i>	333 <i>2.522</i>	1.61 <i>0.207</i>	254 <i>2.405</i>	0.49 <i>0.694</i>	79 <i>1.893</i>	190
Manganese sulphate + sodium nitrate	15.8	2.23 <i>0.349</i>	354 <i>2.549</i>	1.60 <i>0.204</i>	253 <i>2.404</i>	0.63 <i>0.802</i>	101 <i>2.002</i>	130
Sig. diff. for $P < 0.05$	2.0	<i>0.034</i>	<i>0.036</i>	<i>0.068</i>	<i>0.062</i>	<i>0.055</i>	<i>0.073</i>	51
Sig. diff. for $P < 0.01$	2.7	<i>0.056</i>	<i>0.060</i>	<i>0.113</i>	<i>0.103</i>	<i>0.092</i>	<i>0.122</i>	69

nitrogen or improve the colour of these nitrogen-deficient plants (Table 8; Plate 3, Fig. 2). Treatment with combined nitrogen alone increased the percentage nitrogen but did not improve the colour of the sulphur-deficient plants.

Where nitrogen was provided, sulphur markedly improved the colour and increased the growth of non-legumes (Tables 8 and 9; Plates 3 and 4). The darker green colour of the sulphur-treated plants was associated with an increase in the percentage protein nitrogen and a corresponding decrease in the percentage non-protein nitrogen (Tables 8 and 9). The percentage total nitrogen of the dark green sulphur-treated non-legumes was actually less than that of the pale green, sulphur-deficient plants. Tests with diphenylamine showed a marked accumulation of nitrate in the sulphur-deficient plants provided with nitrate nitrogen.

TABLE 8

EFFECTS OF MANGANESE SULPHATE AND NITRIC ACID AT TWO LEVELS OF MOLYBDENUM ON THE YIELD AND NITROGEN CONTENT OF FLAX; EXPERIMENT 4, SOIL B

(Mean transformation values in *italics*)

Treatments	Dry Matter (g.)	Total Nitrogen (%)	Total Nitrogen (mg.)	Protein Nitrogen (%)	Protein Nitrogen (mg.)	Non-protein Nitrogen (%)	Non-protein Nitrogen (mg.)
No Mn, N, or Mo	1.55	1.44	22	1.11	17	0.32	5
		<i>0.158</i>	<i>1.330</i>	<i>1.044</i>	<i>1.223</i>	<i>0.505</i>	<i>0.661</i>
Manganese sulphate	1.50	1.32	20	0.92	14	0.40	6
		<i>0.120</i>	<i>1.288</i>	<i>0.964</i>	<i>1.141</i>	<i>0.597</i>	<i>0.778</i>
Nitric acid	3.05	3.52	107	0.93	28	2.59	79
		<i>0.546</i>	<i>2.029</i>	<i>0.968</i>	<i>1.453</i>	<i>1.413</i>	<i>1.897</i>
Nitric acid + man- gane sulphate	4.10	2.48	102	1.43	59	1.05	43
		<i>0.394</i>	<i>2.002</i>	<i>1.155</i>	<i>1.763</i>	<i>1.020</i>	<i>1.630</i>
Sodium molybdate	1.45	1.13	16	0.87	12	0.26	4
		<i>0.053</i>	<i>1.217</i>	<i>0.940</i>	<i>1.096</i>	<i>0.415</i>	<i>0.602</i>
Sodium molybdate + manganese sulphate	1.75	1.07	19	0.83	15	0.24	4
		<i>0.031</i>	<i>1.273</i>	<i>0.917</i>	<i>1.167</i>	<i>0.381</i>	<i>0.602</i>
Sodium molybdate + nitric acid	3.40	3.70	125	1.19	40	2.51	85
		<i>0.569</i>	<i>2.100</i>	<i>1.076</i>	<i>1.607</i>	<i>1.399</i>	<i>1.931</i>
Sodium molybdate + nitric acid + manga- nese sulphate	5.55	2.48	138	1.70	95	0.77	43
		<i>0.399</i>	<i>2.138</i>	<i>1.229</i>	<i>1.973</i>	<i>0.876</i>	<i>1.623</i>
Sig. diff. for $P < 0.05$	0.83	<i>0.046</i>	<i>0.176</i>	<i>0.082</i>	<i>0.200</i>	<i>0.182</i>	<i>0.213</i>
Sig. diff. for $P < 0.01$	1.17	<i>0.067</i>	<i>0.256</i>	<i>0.121</i>	<i>0.290</i>	<i>0.264</i>	<i>0.309</i>

The results show that the sulphur was needed for nitrogen metabolism within the plant, promoting protein formation. This effect was associated with a marked increase in symbiotic nitrogen fixation by clover on nitrogen-deficient soil, provided the molybdenum deficiency had been remedied.

(f) Nodulation

Treatment with sulphur increased the total weight of nodules, the total number of nodules, the number of nodules per unit length of root, and the

number of large nodules (Tables 4, 6, 7, 10, 11, and 12). Nodulation was particularly restricted where sulphur was deficient in the presence of molybdenum (Tables 6, 10, and 12). The percentage total nitrogen of these plants

TABLE 9

EFFECTS OF SULPHUR APPLIED AS MANGANESE SULPHATE AND POTASSIUM SULPHATE ON THE YIELD, PROTEIN, AND TOTAL NITROGEN CONTENT OF OATS TREATED WITH NITROGEN; EXPERIMENT 6, SOIL A

Treatment*	Dry Matter (g.)	Total Nitrogen (%)	Total Nitrogen (mg.)	Protein Nitrogen (%)	Protein Nitrogen (mg.)	Non-protein Nitrogen (%)	Non-protein Nitrogen (mg.)
No S or Mn	5.60	3.28	184	0.88	49	2.40	135
Potassium sulphate	6.75	2.72	183	1.66	111	1.06	72
Manganese sulphate	9.75	1.93	188	1.08	105	0.85	83
Potassium sulphate + manganese sulphate	10.50	1.88	198	1.18	124	0.70	74
Sig. diff. for $P < 0.05$	0.91	0.19	41	0.22	12	0.22	34
Sig. diff. for $P < 0.01$	1.52	0.34	75	0.40	23	0.41	62

* MnSO_4 applied at sowing; K_2SO_4 applied 20 days before harvest.

was low, but the percentage non-protein nitrogen high (Table 6). Clover deficient in both sulphur and molybdenum was better nodulated than clover deficient in sulphur alone, and frequently as well nodulated as normal plants

TABLE 10

EFFECTS OF SULPHUR AND MOLYBDENUM DEFICIENCIES ON THE YIELD AND NODULATION OF SUBTERRANEAN CLOVER

Deficiency	Experiment 2, Soil B			Experiment 3, Soil C	
	Dry Matter (g.)	Nodules (mg.)	Relative Estimated Weight of Nodules	Dry Matter (g.)	Relative Estimated Weight of Nodules
Normal plants (treated with $\text{MnSO}_4 + \text{Na}_2\text{MoO}_4$)	4.05	25	21	14.85	24
Plants deficient in sulphur	2.60	14	10	5.20	4
Plants deficient in molybdenum	2.15	35	30	11.90	47
Plants deficient in sulphur and molybdenum	2.50	28	25	5.70	8
Sig. diff. for $P < 0.05$	0.86	11	11	1.35	9
Sig. diff. for $P < 0.01$	1.17	15	14	2.00	13

treated with both elements (Tables 6, 10, and 12). At low molybdenum, sulphur had little effect on the yield or percentage total nitrogen, but decreased the percentage non-protein nitrogen and greatly increased the weight and number of nodules.

Combined nitrogen generally decreased the number and weight of nodules, as did molybdenum (Tables 4, 7, and 12). Nodule formation was much more readily inhibited by applied nitrogen where protein formation was inhibited by sulphur deficiency (Table 12). At the levels used combined nitrogen frequently had little effect on nodulation where sulphur was provided.

Sulphur applied shortly before harvest markedly increased symbiotic nitrogen fixation without increasing the number of nodules (Table 11), confirming evidence presented in other sections that symbiotic nitrogen fixation is not limited at low sulphur level by an inadequate number of nodules.

TABLE 11

EFFECTS OF A LATE DRESSING OF SODIUM SULPHATE AT TWO LEVELS OF MOLYBDENUM ON THE YIELD, NITROGEN CONTENT, AND NODULATION OF SUBTERRANEAN CLOVER; EXPERIMENT 8, SOIL B

(Mean transformation values in italics)

	Dry Matter per Pot (g.)	Total Nitrogen (%)	Total Nitrogen (mg.)	Number of Nodules
No S or Mo	3.4	1.74 <i>0.240</i>	59 <i>1.772</i>	277 <i>2.416</i>
Sodium sulphate *	3.1	1.85 <i>0.264</i>	57 <i>1.759</i>	215 <i>2.319</i>
Sodium molybdate	4.3	1.76 <i>0.246</i>	76 <i>1.882</i>	47 <i>1.681</i>
Sodium molybdate + sodium sulphate*	6.2	2.49 <i>0.396</i>	154 <i>2.188</i>	52 <i>1.714</i>
Sig. diff. for $P < 0.05$	1.4	<i>0.114</i>	<i>0.192</i>	<i>0.607</i>
Sig. diff. for $P < 0.01$	1.9	<i>0.189</i>	<i>0.319</i>	<i>0.857</i>

* Na_2SO_4 applied five weeks before harvest.

The evidence indicates that nodulation reflects the nitrogen status of the clover plant as influenced by nitrogen, molybdenum, and sulphur. Deficiency of molybdenum and of combined nitrogen increases the demand for nitrogen and the intensity of nodulation. Deficiency of sulphur decreases the demand for nitrogen and the intensity of nodulation. The plant requirement for nitrogen is not reflected by the percentage total nitrogen in the plant, as deficiency of sulphur decreases the percentage total nitrogen and decreases the response to nitrogen. At the various levels of nitrogen, molybdenum, and sulphur examined in these experiments the demand for nitrogen is closely reflected by the percentage non-protein nitrogen.

IV. DISCUSSION

Deficiency of sulphur for plants in the soils used has been established by the similar response of plants to different forms of sulphur and the negative yield interactions between the different sulphates; by the absence of response to corresponding chlorides; and by the low sulphur content of deficient plants. The value of 0.05 to 0.06 per cent. of sulphur found in untreated subterranean clover is well within the range of deficiency levels quoted by others for plants showing deficiency symptoms (Goodall and Gregory 1947).

Subterranean clover plants deficient in sulphur showed symptoms of nitrogen deficiency with low percentage total nitrogen, and were indistinguishable in these characters from clover plants deficient in molybdenum. Sulphates greatly increased symbiotic nitrogen fixation as did molybdenum. Increase in the percentage nitrogen content of various legumes has been observed to result

TABLE 12

EFFECT OF SODIUM SULPHATE AT TWO LEVELS OF AMMONIUM NITRATE AND SODIUM MOLYBDATE ON THE YIELD AND NODULATION OF SUBTERRANEAN CLOVER; EXPERIMENT 13, ON A SAMPLE OF SOIL A MORE ACUTELY DEFICIENT IN SULPHUR
(Mean transformation values in *italics*)

Treatments	Dry Matter of Tops (g.)	Dry Matter of Roots (g.)	Ratio of Roots to Total Plant	Number of Nodules per 10 cm. of Tap Root	Number of Nodules per 10 cm. of First Order Branch Roots	Number of Nodules per 10 cm. of Second Order Branch Roots	Number of Nodules $\wedge$ 2 mm. in Length	Relative Estimated Weight of Nodules
No S, N, or Mo	1.43	0.93	0.395	6.2	1.87	0.47	5	20
	<i>1.19</i>	<i>0.97</i>		<i>2.11</i>	<i>2.35</i>	<i>1.87</i>	<i>1.95</i>	<i>1.30</i>
Sodium sulphate	3.87	1.27	0.254	6.9	5.87	2.73	16	100
	<i>1.95</i>	<i>1.12</i>		<i>2.14</i>	<i>2.79</i>	<i>2.48</i>	<i>2.29</i>	<i>2.00</i>
Ammonium nitrate	1.70	0.90	0.345	1.5	0.83	0.07	0	3
	<i>1.30</i>	<i>0.95</i>		<i>1.78</i>	<i>2.08</i>	<i>1.66</i>	<i>1.60</i>	<i>0.43</i>
Ammonium nitrate + sodium sulphate	6.30	1.87	0.229	8.0	4.83	2.17	20	96
	<i>2.51</i>	<i>1.36</i>		<i>2.19</i>	<i>2.71</i>	<i>2.41</i>	<i>2.31</i>	<i>1.97</i>
Sodium molybdate	1.50	0.90	0.378	6.0	1.30	0.27	4	10
	<i>1.22</i>	<i>0.95</i>		<i>2.10</i>	<i>2.22</i>	<i>1.82</i>	<i>1.91</i>	<i>1.00</i>
Sodium molybdate + sodium sulphate	9.17	2.13	0.189	8.5	3.80	1.00	42	100
	<i>3.03</i>	<i>1.46</i>		<i>2.22</i>	<i>2.62</i>	<i>2.13</i>	<i>2.66</i>	<i>2.00</i>
Sodium molybdate + ammonium nitrate	1.77	0.90	0.337	2.9	1.70	0.10	1	4
	<i>1.32</i>	<i>0.95</i>		<i>1.91</i>	<i>2.24</i>	<i>1.69</i>	<i>1.68</i>	<i>0.57</i>
Sodium molybdate + ammonium nitrate + sodium sulphate	9.50	2.20	0.188	7.8	2.50	1.13	29	83
	<i>3.08</i>	<i>1.48</i>		<i>2.18</i>	<i>2.46</i>	<i>2.20</i>	<i>2.50</i>	<i>1.90</i>
Sig. diff. for $P < 0.05$	<i>0.24</i>	<i>0.13</i>	0.238	<i>0.22</i>	<i>0.14</i>	<i>0.18</i>	<i>0.29</i>	<i>0.23</i>
Sig. diff. for $P < 0.01$	<i>0.33</i>	<i>0.19</i>	0.330	<i>0.30</i>	<i>0.19</i>	<i>0.25</i>	<i>0.40</i>	<i>0.31</i>

from the use of sulphur-containing fertilizers on deficient soils (Miller 1919; Reimer and Tartar 1919; Olson and St. John 1921; Neller 1926; Powers 1930; Evans and Greaves 1937; Bledsoe and Blaser 1947). Miller (1919) suggested that the increase in nitrogen content of the legume was due to the sulphates stimulating the action of the legume bacteria. Neller (1926) further suggested that sulphate has an indirect effect upon legumes through its direct action or effect upon the nitrogen-fixing organisms.

Sulphates have also been reported to improve the nodulation of legumes (Duley 1916; Walker 1947). However, Wilson (1915, 1917) obtained results

indicating that sulphates in relatively weak concentration inhibit nodulation. Gaw and Soong (1942) reported improved nodulation and yield of peas with calcium and iron sulphates; improved nodulation but no increase in yield with sodium and zinc sulphates; and decreased nodulation with other sulphates, including potassium sulphate. Anmonium sulphate increased the yield but not nodulation.

In the present experiments increase in the yield of clover due to sulphur was associated with an increase in both the number and size of nodules. However, a late application of sulphate caused a rapid increase in nitrogen fixation without any increase in the number of nodules. In addition, clover deficient in sulphur remained unthrifty when provided with combined nitrogen. This contrasts sharply with the effect of nitrogen on clover with insufficient molybdenum for optimum symbiotic nitrogen fixation. The results show that in sulphur deficiency the growth of the host plant was not limited by defective nodulation or by ineffective symbiotic nitrogen fixation.

The need of the clover for sulphur in the host plant is indicated by the strong positive interaction between sulphur and nitrogen on clover deficient in molybdenum for symbiotic nitrogen fixation. This interaction contrasts with the negative interaction between molybdenum and nitrogen on legumes (Anderson and Thomas 1946; Anderson and Spencer 1950).

Extensive studies on the influence of carbohydrate and nitrogen on nodulation have indicated that invasion of the plant by *Rhizobium*, as measured by the number of nodules, increases as the carbohydrate-nitrogen relationship is widened, irrespective of the method used to alter the relationship (Wilson 1940). The results have shown a definite negative correlation between the percentage nitrogen in the plant and the number of nodules. In the present experiments, molybdenum (through symbiotic nitrogen fixation), combined nitrogen, and sulphur all increase the percentage total nitrogen content of the clover, and might therefore be expected to have similar effects on the carbohydrate-nitrogen ratio. It is, in fact, well established that plants provided with combined nitrogen but deficient in sulphur have a high percentage of carbohydrate and a low percentage of protein (Nightingale, Schemerhorn, and Robbins 1932; Heiserich 1935; Eaton 1935, 1941, 1942).

Increase in percentage nitrogen induced by molybdenum or combined nitrogen resulted in a decrease in nodulation. However, sulphur increased the percentage total nitrogen and improved nodulation. This would normally be expected where improved nodulation was the cause and not the result of increased nitrogen fixation. The experiments have shown conclusively, however, that the sulphur-deficient clover plants were not deficient in nitrogen for protein formation or growth.

Deficiencies of sulphur and nitrogen would, however, be expected to have opposite effects on the non-protein nitrogen content of the plants. Determination of non-protein nitrogen in clover showed that plants deficient in sulphur contained a higher content of this nitrogen fraction. Deficiencies of molybdenum and nitrogen decreased the non-protein nitrogen content of the clover.

Hence decrease in the number of nodules was associated in all cases with an increase in the percentage non-protein nitrogen. The effect of sulphur and molybdenum on nodulation was evident even where deficiency of either one of the elements almost entirely prevented an effect of the other on growth or protein content. The sulphur-molybdenum interaction on yield and protein content was positive and highly significant. Clover receiving sulphur but deficient in molybdenum was low in non-protein nitrogen and usually had abnormally large numbers of nodules compared with normal plants provided with both elements. Clover deficient in sulphur but provided with molybdenum was high in non-protein nitrogen and had restricted nodulation.

While the effects of molybdenum, nitrogen, and sulphur on the number of nodules in these experiments were inversely related to the effects on the non-protein nitrogen content of the clover, it cannot be assumed that the non-protein nitrogen inhibited nodule formation. It is, however, not impossible, for example, that some soluble nitrogen compound is involved in increasing the resistance of the root hair to curling normally induced by the bacterial secretions. It is known that curling of the root hairs can be prevented by combined nitrogen (Thornton 1935). On the other hand Thornton was able to prevent this effect of nitrogen by simultaneously providing the plant with a soluble carbohydrate. The present experiments with sulphur are not necessarily inconsistent with evidence that a carbohydrate is involved. In this connection it is interesting to note that in some cases percentage sugars are reported to have been decreased by sulphur deficiency, while the percentage of starch and other carbohydrates was increased (Eaton 1935, 1941). Eaton (1942) found that sulphur deficiency decreased the content of reducing sugars, but increased the content of sucrose in black mustard. Nightingale, Schermerhorn, and Robbins (1932) found that translocation of sugars occurred freely in tomato plants deficient in sulphur, and Heiserich (1935) reported a high concentration of sugars in sulphur-deficient maize and tobacco.

The evidence presented shows that conditions in the clover favouring nodulation are not necessarily inversely related to the percentage nitrogen content of the clover, and are in fact positively correlated with sulphur deficiency. Thus the effect of sulphur on nodulation cannot be explained simply in terms of the carbohydrate-nitrogen hypothesis. Decrease in nodulation brought about by sulphur deficiency is associated with an increase in percentage carbohydrate, suggesting that carbohydrate supply may not be directly involved in regulating nodule formation.

Non-legumes in the present experiments required treatment with sulphur and combined nitrogen for protein formation and normal growth, and were pale green when deficient in either sulphur or nitrogen. The nitrogen-sulphur interaction on dry matter and protein was positive as was the case for legumes with insufficient molybdenum for symbiotic nitrogen fixation. Similar effects on yield were obtained by Tolman and Stoker (1941), who found a positive nitrogen-sulphur interaction with sugar beet.

It is well established that increase in protein content of plants is associated with a darker green colour, and the similarity of symptoms of sulphur and nitrogen deficiency has frequently been noted in the literature. Mandels (1943) found that chlorosis in *Chlorella* sp. was induced by sulphur deficiency through a decrease in the amount of chlorophyll per cell before cell division stops, and by a slow decomposition of the chlorophyll in the non-dividing cells. Hanson, Barrien, and Wood (1941) found that chloroplast proteins are rich in sulphur, and that the ratio chloroplast protein nitrogen: chloroplast protein sulphur remained approximately constant during the life cycle. Wood and Barrien (1939) had earlier shown that the formation of protein sulphur from sulphate is dependent upon the nitrogen supply.

The effect of sulphur in increasing the percentage protein nitrogen of non-legumes at the expense of the non-protein nitrogen is similar to the effect of molybdenum (Anderson and Spencer 1949, 1950). In addition, sulphur deficiency, like molybdenum deficiency, resulted in a marked accumulation of nitrate in plants provided with nitrate nitrogen. Like molybdenum, sulphur did not increase the percentage total nitrogen of non-legumes.

The observed accumulation of nitrate in plants deficient in molybdenum has been used as evidence that molybdenum is directly involved in a nitrate-reducing system. Accumulation of nitrate may occur also in plants deficient in manganese (Leeper 1941), and Burstrom (1945) suggests that a manganese-containing system promotes the reduction of nitrate to ammonia. However, Hewitt, Jones, and Williams (1949) obtained evidence that manganese is required for protein formation from amino-acids already formed. There is evidence that molybdenum is needed for the utilization of ammonium or amino acid nitrogen (Bortels 1939; Steinberg 1942; Vanselow and Datta 1949). Wood (1942) has concluded that data on sulphur metabolism suggests that nitrate and sulphate reduction are linked in a complex way with carbohydrate utilization and with each other, but that the mechanism remains obscure. It is interesting to note that nitrate accumulation may occur in plants deficient in sulphur, manganese, or molybdenum, and that all three elements are needed for protein formation.

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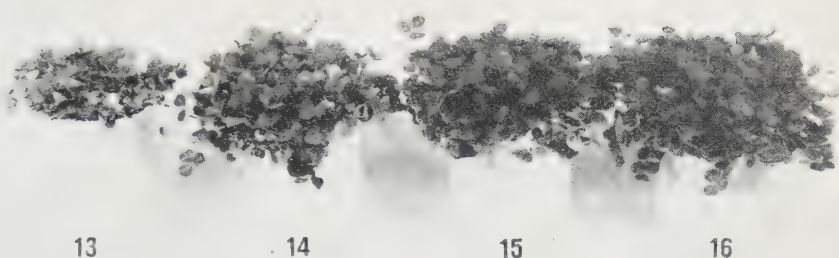


Fig. 1.—Interaction between two forms of sulphur on subterranean clover (Experiment 9). (13) No S or Mo. (14) 3 cwt. MnSO_4 per acre (58 mg. S per pot). (15) K_2SO_4 (58 mg. S per pot). (16) MnSO_4 + K_2SO_4 , each at levels as above.

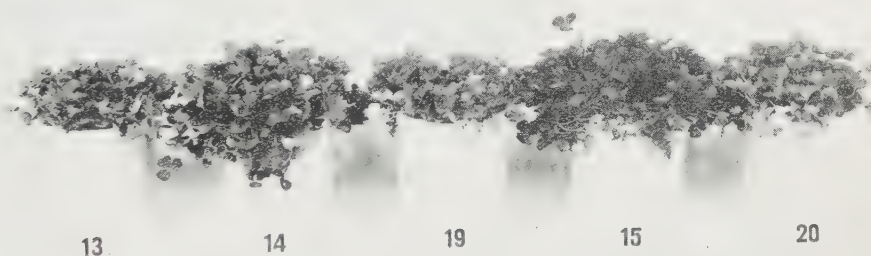


Fig. 2.—Effects of sulphates and chlorides of manganese and potassium on subterranean clover (Experiment 9). (13) No S or Cl. (14) 3 cwt. MnSO_4 per acre (100 mg. Mn + 58 mg. S per pot). (19) MnCl_2 (100 mg. Mn per pot). (15) K_2SO_4 (58 mg. S per pot + 141 mg. K per pot). (20) KCl (141 mg. K per pot).

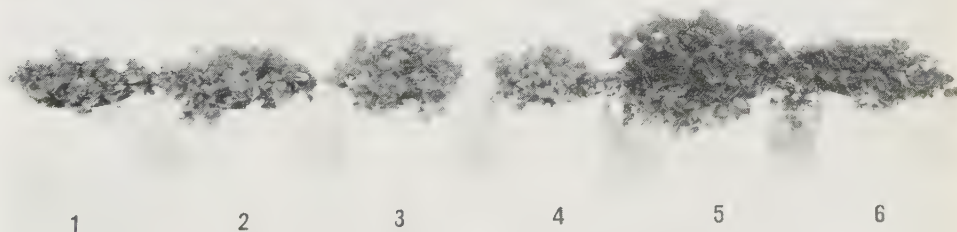


Fig. 3.—Effects of nitrogen and of ammonium sulphate on sulphur-deficient subterranean clover (Experiment 10). The nitric acid provided 303 mg. N per pot, and other nitrogen nutrients 289 mg. N per pot. (1) No N or S. (2) Sodium nitrate. (3) Nitric acid. (4) Ammonium chloride. (5) Ammonium sulphate. (6) Urea.

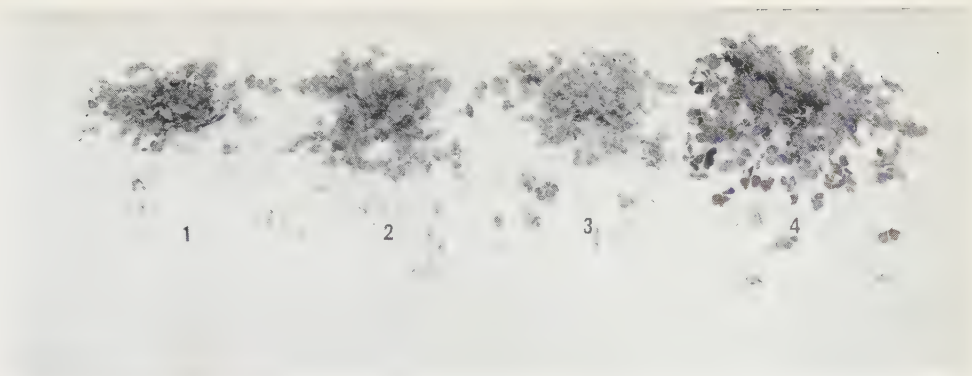


Fig. 1.—Molybdenum-sulphur interaction on subterranean clover (Experiment 1). (1) No Mo, Mn, or S. (2) 1 lb. Sodium molybdate per acre. (3) 5 cwt. Manganese sulphate per acre. (4) 1 lb. Sodium molybdate + 5 cwt. manganese sulphate per acre.

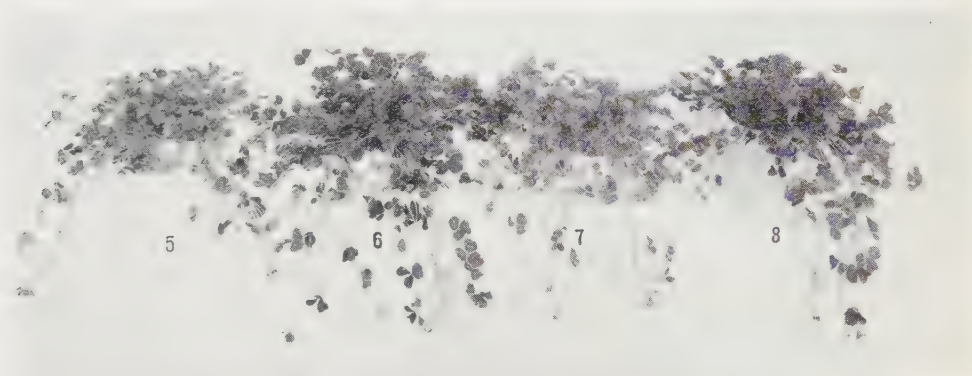


Fig. 2.—Effects of molybdenum and sulphur treatments described for Figure 1 on subterranean clover provided with ammonium sulphate (Experiment 1). (5) No Mo, Mn, or S. (6) Sodium molybdate. (7) Manganese sulphate. (8) Sodium molybdate + manganese sulphate.

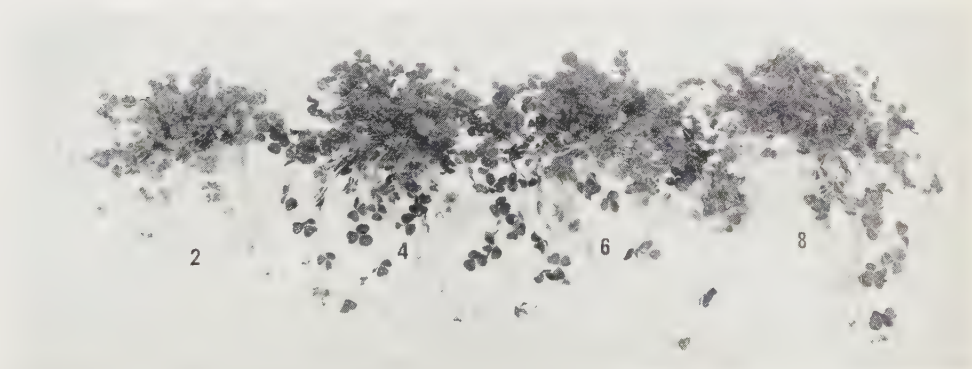


Fig. 3.—Interaction between sulphur provided as ammonium sulphate and manganese sulphate on subterranean clover in the presence of molybdenum (Experiment 1). (2) No S. (4) 5 cwt. Manganese sulphate per acre. (6) 7 cwt. Ammonium sulphate per acre. (8) 5 cwt. Manganese sulphate + 7 cwt. ammonium sulphate per acre.



Fig. 1.—Effect of manganese chloride and manganese sulphate on oats provided with nitrogen as nitric acid (Experiment 7). (13) No Mn. (14) 1 cwt. Manganese chloride per acre. (15) 5 cwt. Manganese sulphate per acre. (16) 1 cwt. Manganese chloride + 5 cwt. manganese sulphate per acre.



Fig. 2.—Showing the nitrogen-sulphur interaction on paspalum (Experiment 11). (1) No N or S. (5) 5 cwt. Ammonium chloride per acre. (3) 132 lb. Potassium sulphate per acre. (6) 5 cwt. Ammonium chloride + 132 lb. potassium sulphate per acre.



Effect of sulphur deficiency on flax (Experiment 6).
Left.—Sulphur deficient.
Right.—With 5 cwt. manganese sulphate per acre.

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RELATION BETWEEN NITROGEN SOURCE AND THE EFFECTS ON FLAX OF AN EXCESS OF MANGANESE OR MOLYBDENUM IN THE NUTRIENT SOLUTION

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Summary

The effects on flax of a given concentration of manganese or molybdenum in the nutrient solution were found to depend on the amount and form of nitrogen supplied. The interactions that occurred between either of these two elements on the one hand, and nitrogen source on the other, were highly significant but opposite in effect.

Thus, with a given excess of manganese, symptoms were most severe in plants grown in solution supplied with nitrogen as nitrate or urea, and were absent in nitrogen-deficient solutions or those supplied with nitrogen as ammonium. In solutions supplied with ammonium nitrate, no manganese toxicity symptoms occurred or these were only slight. A concentration of 50 p.p.m. of manganese in the solution caused a very significant decrease in growth in the presence of nitrate, had no harmful effect on growth in the presence of ammonium nitrate, and caused a significant increase in growth with ammonium. With urea, the reduction in growth was not as great as with nitrate. The presence of ammonium ions in the solution markedly reduced the manganese concentration in the plants when compared with that of plants receiving nitrate only.

By contrast, at a given excess of molybdenum in the nutrient solution, toxicity symptoms in the test plants were affected by nitrogen source in the following order of decreasing severity: nitrogen deficiency > ammonium > ammonium nitrate > urea > nitrate. In solutions supplied with nitrate, a given concentration of molybdenum was significantly more toxic to growth when applied as ammonium molybdate than as sodium molybdate. In one experiment the addition to the nitrate solution of 10 p.p.m. of molybdenum as sodium molybdate resulted in a significant increase in the dry weight of the plants compared with the controls, even though such plants contained 400 p.p.m. of molybdenum on a dry basis. In the presence of ammonium or urea, no significant differences occurred between the effects of sodium molybdate or ammonium molybdate on plant growth.

In the nitrate nutrient solutions less molybdenum was absorbed by the plants when a given excess of this element was added to the solution as sodium molybdate than when it was added as ammonium molybdate. It is concluded that the toxicity to flax of a given concentration of molybdenum in the nutrient solution is increased by the presence of ammonium (NH_4^+) ions.

Where excess of manganese and molybdenum were added together to the nutrient solution, an interaction between the two elements occurred. Thus the severity of manganese-induced iron-deficiency chlorosis and lower leaf necrosis was reduced. However, a golden-yellow chlorosis, not typical of iron-deficiency chlorosis but similar to molybdenum toxicity symptoms, was induced in the

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plants in the presence of nitrate, ammonium nitrate, or urea, but not where ammonium was the sole source of nitrogen. The manganese-molybdenum interaction was facilitated by the presence of some ammonium ions in the solution. Thus, a given concentration of molybdenum was more effective as ammonium molybdate than as sodium molybdate in reducing the severity of manganese toxicity symptoms. However, the addition to the nitrate solution of ammonium in an amount equivalent to ammonium molybdate had no effect on manganese toxicity symptoms. Excess boron (2 p.p.m.) had no effect on manganese toxicity symptoms.

When grown in nutrient solutions containing nitrate, flax plants affected with manganese toxicity symptoms had a higher nitrate content than normal. Manganese-molybdenum antagonism (i.e. reduction in severity of manganese-induced iron-deficiency chlorosis and lower leaf necrosis in the presence of excess molybdenum), was associated in this instance with a reduction in the nitrate content of the plants. However, when grown in nutrient solutions containing urea, no nitrate was detected in plants showing severe manganese toxicity symptoms, although, in this instance also, the severity of the leaf necrotic symptoms was reduced by adding molybdenum.

I. INTRODUCTION

It has been recognized in the literature that plants growing on substrates containing excessive amounts of manganese will develop a chlorosis identical with that of iron deficiency, and can be cured by supplying additional iron to the plant. Characteristic manganese-induced necrotic symptoms also occur in the older leaves of the plants—usually the middle leaves first show these symptoms. These excess-manganese symptoms in flax have been described by Millikan (1947, 1949*b*). The lower leaf necrosis is unrelated to iron deficiency, as it occurred in flax plants in which the development of symptoms of iron deficiency chlorosis had been prevented by supplying additional iron, grown in nutrient solutions containing excess manganese.

An excess of molybdenum in the substrate may produce a golden-yellow coloured chlorosis, particularly in the older leaves of the flax plant (Millikan 1949*b*). This chlorosis is different in colour from that of manganese-induced iron deficiency, and is not prevented by supplying additional iron.

The water culture experiments described below have been conducted to determine the effects of nitrogen deficiency, or of nitrogen supplied as nitrate, ammonium nitrate, ammonium, or urea respectively, on the toxicity to plants of an excess of manganese or molybdenum, or both, in the nutrient solution.

II. METHOD

The nutrient solution containing nitrate used was that of Arnon (1938) and included the A4 and B7 solutions of trace elements. In the solutions containing ammonium sulphate, ammonium nitrate, or urea respectively, or the solution deficient in nitrogen, the calcium nitrate of Arnon's solution was replaced by calcium sulphate and potassium nitrate by potassium sulphate. The total amount of nitrogen was the same in all solutions containing this element.

In the first three experiments, the pH of the solution was adjusted to pH 4.5 at least once a week, but in subsequent experiments regular bi-weekly adjustments of the pH were made. The iron solution contained 0.5 per cent. ferrous sulphate and 0.4 per cent. tartaric acid, and was added twice at the rate of 0.6 ml. per litre of culture solution during the first week of growth, and thereafter at weekly intervals. The plants were transferred to fresh solutions after approximately three to four weeks.

The actual concentrations of manganese or molybdenum applied to each series of cultures containing the different levels or forms of nitrogen are set out in the various tables below. The manganese was applied as $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and the molybdenum as $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (designated as $\text{Mo}(\text{NH}_4)$) or $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (= $\text{Mo}(\text{Na})$) respectively. Cultures containing excesses of both manganese and molybdenum were included. In the first five experiments each treatment was duplicated, but in the final three experiments, five, six, and ten replications respectively were employed.

The solutions were contained in two-litre pyrex glass beakers painted on the outside with gold size followed by two coats of black paint. The tops for the beakers were made of plaster of paris soaked in hot paraffin wax. The test plants were inserted through conveniently sized and spaced holes drilled through the tops.

The flax was germinated in acid-washed sand and transferred to the water cultures as soon as possible after emergence. In the first three experiments twelve plants were grown per beaker, but this number was reduced to eight in subsequent experiments.

All experiments were conducted out of doors, and have been repeated at different seasons of the year. During the course of the experiments, tests were made on middle leaves of the plants for the presence of nitrates and other oxidizing materials, by the use of 1 per cent. diphenylamine in concentrated sulphuric acid (Thornton, Conner, and Fraser 1939).

III. RESULTS

In general it was found that, after a few weeks, growth of plants in the control ammonium solutions was much inferior to that of plants in the control nitrate, urea, or ammonium nitrate solutions. Best growth occurred in the last solution. The ammonium solutions became more acid as growth occurred, whereas the nitrate and urea solutions became more alkaline. This phenomenon with respect to nitrate and ammonium has been observed previously by Arnon (1937).

The following are the detailed results of the various experiments made.

(a) Experiment 1

In the first experiment the effects on flax grown therein of the addition of various levels of manganese to nutrient solutions containing either nitrate or ammonium as the source of nitrogen were studied. The manganese toxicity symptoms that occurred are shown in Table 1.

In the nutrient solutions supplied with nitrate, excess manganese induced severe symptoms of iron-deficiency chlorosis and lower leaf necrosis (see Plate 1, Fig. 1) in the plants grown therein. These symptoms were identical with those of manganese toxicity in flax described by Millikan (1949*b*) and referred to above. However, where ammonium was the source of nitrogen, the same concentrations of manganese in the nutrient solution induced no symptoms in the plants.

TABLE 1

MANGANESE TOXICITY SYMPTOMS* IN THE AERIAL PARTS OF FLAX PLANTS AFTER 25 DAYS' GROWTH IN NITRATE OR AMMONIUM NUTRIENT SOLUTIONS CONTAINING EXCESS MANGANESE. EXPERIMENT 1, MAY 1948

Treatment †	Nitrogen Source	
	NO ₃	NH ₄
Control	—	—
Mn 10 p.p.m.	—	—
Mn 20 p.p.m.	C ++	—
Mn 50 p.p.m.	C +++ N ++	—
Mn 100 p.p.m.	C +++ N ++	—

* C = iron-deficiency chlorosis. N = manganese-induced necrosis of the older leaves commencing at the tips. Symptom intensity: — no symptoms, + slight, ++ moderate, +++ severe, ++++ very severe.

† Manganese applied as MnSO₄·4H₂O.

(b) Experiment 2

The nitrogen sources studied in the second experiment were nitrate, ammonium nitrate, and ammonium respectively. Excesses of manganese, molybdenum, or both, were added to the solutions containing each nitrogen source. The symptoms that developed in the plants are recorded in Table 2 together with the results of nitrate tests on the plants after three weeks' growth.

This experiment also demonstrated that the effect on flax of a given concentration of manganese in the nutrient solution was dependent on the nitrogen source in that solution (see Plate 1, Fig. 2). Whereas severe symptoms occurred in plants grown in the nitrate solution, the same concentrations caused no symptoms in the ammonium solution and only slight symptoms at the highest manganese level in the ammonium nitrate solution after five weeks.

The addition of molybdenum in conjunction with the manganese delayed the appearance of manganese toxicity symptoms in the plants, and also reduced their final severity. A very slight chlorotic interaction effect between the manganese and molybdenum was observed in this experiment in the presence of ammonium nitrate. This interaction was more marked in subsequent experiments.

TABLE 2
SYMPTOMS* IN AERIAL PARTS OF FLAX PLANTS AFTER THREE AND FIVE WEEKS' GROWTH
IN NUTRIENT SOLUTIONS, AND RESULTS OF NITRATE TEST† AT THREE WEEKS.
EXPERIMENT 2, AUGUST-SEPTEMBER 1948

Treatment‡	Nitrogen Source									
	NO ₃			NH ₄ NO ₃			NH ₄			
	Nitrate Test	Three Weeks	Five Weeks§	Nitrate Test	Three Weeks	Five Weeks§	Nitrate Test	Three Weeks	Five Weeks§	
Control	+	-	-	+	-	-	-	-	-	-
Mn 20 p.p.m.	+++	C++	C++	+	-	-	-	-	-	-
Mn 50 p.p.m.	+++	C+++	C+++	+	-	-	-	-	-	-
Mn 100 p.p.m.	++++	C+++	C+++ N+++	-	-	N++	-	-	-	-
Mn 20 p.p.m. + Mo 10 p.p.m.	-	-	C++	-	-	-	-	-	-	-
Mn 50 p.p.m. + Mo 10 p.p.m.	-	-	C++	-	-	-	-	-	-	-
Mn 50 p.p.m. + Mo 20 p.p.m.	-	-	C++	-	-	-	-	-	-	-
Mn 100 p.p.m. + Mo 10 p.p.m.	-	C-	C++	-	-	-	-	-	-	-
Mn 100 p.p.m. + Mo 20 p.p.m.	-	-	C++	-	-	C+	-	-	-	-
Mo 10 p.p.m.	-	-	-	-	-	-	-	-	-	-
Mo 20 p.p.m.	-	-	-	-	-	-	-	-	-	-

* See footnote to Table 1. † Reagent used 1 per cent. diphenylamine in concentrated sulphuric acid. Intensity of reaction: - nil, + very slight, ++ slight, +++ moderate, ++++ marked. ‡ Manganese applied as MnSO₄·4H₂O, molybdenum as (NH₄)₆Mo₇O₂₄·4H₂O.

§ Solutions renewed after three weeks.

The occurrence of manganese toxicity symptoms in the plants in the nitrate solutions was associated with a marked increase in the nitrate content of such plants (Plate 2, Fig. 1).

TABLE 3
SYMPTOMS* IN THE AERIAL PARTS OF FLAX PLANTS AFTER 15 AND 25 DAYS' GROWTH IN NUTRIENT SOLUTIONS. EXPERIMENT 3, SEPTEMBER-OCTOBER 1948

Treatment†	Nitrogen Source					
	NO_3		NH_4NO_3		Nitrogen Deficiency	
	15 Days	25 Days	15 Days	25 Days	15 Days	25 Days
Control	—	—	—	—	—	—
Mn 20 p.p.m.	C+	C+++	—	—	—	—
Mn 50 p.p.m.	C+++	C++++	—	—	—	—
Mn 100 p.p.m.	C+++	C++++ N++	—	—	—	—
Mn 20 p.p.m. + Mo 10 p.p.m.	—	G+++	—	—	—	—
Mn 20 p.p.m. + Mo 20 p.p.m.	—	G+++	—	G++	—	—
Mn 20 p.p.m. + B 2 p.p.m.	C++	C+++	—	—	—	—
Mn 50 p.p.m. + Mo 10 p.p.m.	—	G++++	—	G+	—	—
Mn 50 p.p.m. + Mo 20 p.p.m.	—	G++++	—	G++	—	—
Mn 50 p.p.m. + B 2 p.p.m.	C++	C++++	—	—	—	—
Mn 100 p.p.m. + Mo 10 p.p.m.	—	G++++	—	G++	—	—
Mn 100 p.p.m. + Mo 20 p.p.m.	—	G++++	—	G+++	—	—
Mn 100 p.p.m. + B2 p.p.m.	C+++	C++++ N++	—	—	—	—
Mo 10 p.p.m.	—	—	—	—	—	—
Mo 20 p.p.m.	—	G+	—	—	—	—
B 2 p.p.m.	—	—	—	—	—	—

* C = iron-deficiency chlorosis. G = golden-yellow colour most marked in tips of older leaves. N = manganese-induced necrosis of older leaves commencing at the tips. Symptom intensity: — nil, + slight, ++ moderate, +++ severe, ++++ very severe.

† See footnote to Table 2. Boron applied as H_3BO_3 .

(c) Experiment 3

The general design of this experiment was similar to that of Experiment 2 except that the ammonium series was substituted by a nitrogen-deficient treatment. The effect of boron (2 p.p.m.) on the development of manganese toxicity symptoms under each nitrogen treatment was also studied. Schappele (1946)

TABLE 4
SYMPTOMS^a IN AERIAL PARTS OF FLAX PLANTS AFTER 10, 17, AND 25 DAYS' GROWTH IN
NUTRIENT SOLUTIONS. EXPERIMENT 4, OCTOBER-NOVEMBER 1948

Treatment†	Nitrogen Source									
	NO ₃			NH ₄ NO ₃			NH ₄			
	10 Days	17 Days†	25 Days†	10 Days	17 Days†	25 Days†	10 Days ^s	17 Days†	25 Days†	
Control	-	-	-	-	-	-	-	-	-	-
Mn 20 p.p.m.	-	G++	G+++	-	-	-	-	-	-	-
Mn 50 p.p.m.	G+++	G+++	G+++	-	-	-	-	-	-	-
Mn 100 p.p.m.	G+++	G+++	N++ G+++	-	-	-	-	-	-	-
Mn 20 p.p.m. + Mo(NH ₄) 10 p.p.m.	-	-	G+++	-	-	-	-	-	-	-
Mn 20 p.p.m. + Mo(NH ₄) 20 p.p.m.	-	-	G+++	-	-	-	-	-	-	-
Mn 20 p.p.m. + Mo(Na) 10 p.p.m.	G+++	G+++	G+++	-	-	-	-	-	-	-
Mn 50 p.p.m. + Mo(NH ₄) 10 p.p.m.	-	G++	G+++	-	-	-	-	-	-	-
Mn 50 p.p.m. + Mo(NH ₄) 20 p.p.m.	-	-	G+++	-	-	-	-	-	-	-
Mn 50 p.p.m. + Mo(Na) 10 p.p.m.	G+++	G+++	G+++	-	-	-	-	-	-	-
Mn 100 p.p.m. + Mo(NH ₄) 10 p.p.m.	-	G++	G+++	-	-	G+++	-	-	-	-
Mn 100 p.p.m. + Mo(NH ₄) 20 p.p.m.	-	-	G+++	-	-	G+++	-	-	-	-
Mn 100 p.p.m. + Mo(Na) 10 p.p.m.	G+++	G+++	G+++	-	-	-	-	-	-	-

^a See footnotes to Table 3. † Treatments applied as follows: Mn as MnSO₄·4H₂O, Mo(NH₄) as (NH₄)₆Mo₇O₂₄·4H₂O, Mo(Na) as Na₂MoO₄·2H₂O. ‡ Solutions renewed after 14 days.

has reported that the addition of boron counteracted the chlorosis associated with manganese toxicity in pineapples grown in water cultures.

Manganese toxicity symptoms were again found to be markedly influenced by nitrogen source (see Table 3). They were absent in the plants subjected to nitrogen deficiency and those receiving ammonium nitrate. By contrast, with nitrate as the source of nitrogen supply, severe symptoms of manganese-induced iron-deficiency chlorosis, and, at the highest manganese level (100 p.p.m.), moderate symptoms of lower leaf necrosis occurred.

By comparison with the plants receiving excess manganese alone, the addition of excess molybdenum to the solutions, in conjunction with excess manganese, delayed the appearance of any symptoms in the plants in the nitrate series, but after 25 days this treatment had induced a marked golden-yellow chlorosis in the plants. This golden-yellow chlorosis was different in colour from iron-deficiency chlorosis and was also not typical of the latter in that it involved all the leaves of the plants, and was most apparent in the tips of the lower leaves (Plate 3, Fig. 2). This symptom appeared similar to that of molybdenum toxicity described by Millikan (1949). The golden-yellow interaction effect between manganese and molybdenum was also apparent to a lesser degree in the ammonium nitrate series, even though the plants in this series which received an excess of manganese or molybdenum alone showed no chlorosis. However, even though the golden-yellow interaction occurred, the addition of molybdenum in conjunction with the highest level of manganese prevented the occurrence of lower leaf necrosis in the plants in the nitrate series.

By contrast, excess boron applied in conjunction with excess manganese had no effect on the development or appearance of manganese toxicity symptoms in the plants so treated.

(d) Experiment 4

The design of this experiment was similar to that of Experiment 2, except that a sodium molybdate treatment was added at each manganese level. The results in Table 4 indicate that, in this experiment also, no symptoms of manganese toxicity occurred in the plants receiving an excess of this element in the ammonium or ammonium nitrate series, whereas severe symptoms occurred in the presence of excess manganese where nitrate was the sole source of nitrogen.

In the nitrate series ammonium molybdate applied in conjunction with excess manganese delayed the appearance of any symptoms, although the golden interaction effect, observed in Experiment 3, finally occurred in the plants. This golden-yellow chlorosis occurred also in the ammonium nitrate series (only at the manganese 100 p.p.m. level) but not in the ammonium series.

By contrast, sodium molybdate was found to be ineffective in delaying the appearance of chlorosis at all manganese levels in the nitrate series. However, the chlorosis had the typical golden-yellow colour of the manganese-molybdenum interaction effect. Both forms of molybdenum prevented the occurrence of lower leaf necrosis. It was observed that, in the nitrate solution,

a given concentration of molybdenum was less toxic to the growth of the plants as sodium molybdate than as ammonium molybdate, whereas in the presence of ammonium or ammonium nitrate these two molybdenum sources appeared to be equally toxic.

As growth occurred it was found that, in the solution containing ammonium molybdate, the pH tended to remain constant or become more acid, whereas when sodium molybdate was present the solution became more alkaline. It appeared evident that the ammonium content of the ammonium molybdate was the factor causing the above differences in the effects of the two molybdenum sources.

(e) *Experiment 5*

In view of the differences in the effects of ammonium molybdate and sodium molybdate reported in Experiment 4, a further experiment was made to investigate the effects on flax plants of the addition of an amount of ammonium equivalent to that in ammonium molybdate to nitrate nutrient solutions containing various levels of excess manganese. The ammonium was added with and without sodium molybdate, and two levels of iron supply were included. The symptoms that occurred in the plants are presented in Table 5.

In this experiment ammonium molybdate at the highest concentration used (20 p.p.m.), when added to the solution in conjunction with excess manganese, caused a marked reduction in manganese toxicity symptoms of chlorosis and lower leaf necrosis. By contrast, an equivalent concentration of ammonium as ammonium sulphate added with excess manganese had no effect on the severity of such symptoms. However, when applied with sodium molybdate, ammonium caused a reduction in lower leaf necrosis at the lowest excess manganese level only.

The golden-yellow interaction effect recorded in previous experiments was most marked in the presence of excess manganese and sodium molybdate, whereas excess manganese alone induced typical iron-deficiency chlorosis and excess sodium molybdate alone produced no chlorosis. Except at the lower excess manganese levels, sodium molybdate had no effect on the severity of manganese-induced lower leaf necrosis.

Twice the normal iron supply caused a reduction in the severity of typical iron-deficiency chlorosis except at the 100 p.p.m. Mn level, but had no effect on lower leaf necrosis. Increase in iron level had no consistent effect on the golden-yellow chlorosis.

The results of the tests for nitrate indicated that manganese-induced iron-deficiency chlorosis and the golden-yellow interaction effect were both associated with an increased nitrate content in the plant, whereas the prevention of chlorosis in the presence of ammonium molybdate was associated with the complete absence of nitrate. As in Experiment 4, it was again observed that a given concentration of molybdenum was much less toxic to growth in the nitrate solution as sodium molybdate than as ammonium molybdate (see Plate 2, Fig. 2).

TABLE 6
SYMPTOMS* IN AERIAL PARTS OF PLANTS, AND RESULTS OF NITRATE TESTS† ON LEAVES,
AFTER 30 DAYS' GROWTH IN WATER CULTURE. EXPERIMENT 6, OCTOBER-NOVEMBER 1949

Treatment†	Nitrogen Source							
	NO ₃		NH ₄ NO ₃		NH ₄		Urea	
	Symptoms	Nitrate Test	Symptoms	Nitrate Test	Symptoms	Nitrate Test	Symptoms	Nitrate Test
Control	-	++	-	-	-	-	-	-
Mn 50 p.p.m.	C+++ N+++	+++	C+	-	-	-	C++ N+++	-
Mo(NH ₄) 10 p.p.m.	-	-	-	-	-	-	-	-
Mo(NH ₄) 20 p.p.m.	G-	++	-	-	G+	-	G+	-
Mo(Na) 10 p.p.m.	-	-	-	-	-	-	-	-
Mo(Na) 20 p.p.m.	-	+	-	-	-	-	G++	-
Mn 50 p.p.m. + Mo(NH ₄) 20 p.p.m.	G++++	+	G+++	+	-	-	G+++	-
Mn 50 p.p.m. + Mo(Na) 20 p.p.m.	G++++	++	G++++	+	-	-	G++++	-
Mn 50 p.p.m. + Mo(Na) 20 p.p.m. NH ₄ (≡ Mo(NH ₄)) 20 p.p.m.)	G++++	+	-	-	-	-	-	-

* See footnotes to Table 3.

† See footnotes to Table 2. Solutions renewed after 3 weeks.

‡ See footnotes to Table 4.

(f) Experiment 6

The previous experiments had shown that a marked interaction existed between the nitrogen source in the nutrient solution and the effects on the flax plants of the addition of an excess of manganese to that solution. Indications were also obtained of the existence of a reverse interaction between nitrogen source and molybdenum. An experiment was therefore conducted to obtain quantitative data on the relative effects of applications of manganese or molybdenum, or both, to solutions containing different sources of nitrogen on the growth (dry weight) of the plants. The molybdenum and manganese contents of the plants were also determined. Four nitrogen sources, namely nitrate, ammonium nitrate, ammonium, and urea, were included in the experiment and each treatment was replicated five times.

The results of this experiment are presented in Tables 6, 7, and 8. The symptom picture (Table 6) conformed with that of previous experiments. Manganese toxicity symptoms were severe in the nitrate, slight in the ammonium nitrate, and absent in the ammonium solutions respectively. With urea as the nitrogen source the severity of manganese toxicity symptoms was comparable with that in the nitrate series. The golden-yellow interaction effect between manganese and molybdenum occurred in the nitrate, ammonium nitrate, and urea series, but not in the ammonium series. However, no manganese-induced lower leaf necrosis occurred in any of the plants showing this interaction effect. A similar golden-yellow chlorosis occurred to a slight degree in the presence of molybdenum alone in the presence of the nitrogen sources nitrate, ammonium, and urea respectively.

Nitrate tests showed a slight increase in the presence of manganese in the nitrate series and a reduction in the presence of molybdenum. The only nitrate detected in the remaining plants in the experiment was a very slight reaction in the ammonium nitrate series where manganese and molybdenum were supplied jointly, and where the golden-yellow chlorosis occurred.

A statistical examination of the growth (dry weight) data of the plants shown in Table 7 showed that highly significant interactions occurred between nitrogen source and manganese or molybdenum treatment. Manganese was most toxic to growth in the nitrate solution and significantly less toxic in the presence of urea as the nitrogen source. No toxic effect occurred in either the ammonium nitrate or ammonium solutions—in fact, top growth was actually increased, but not significantly so, by the addition of 50 p.p.m. of manganese, and root growth was significantly increased by this treatment in the ammonium nitrate solution.

The highly significant interaction between molybdenum and nitrogen sources was the reverse of that which occurred with manganese. Thus the arrangement of the nitrogen sources in order of increasing molybdenum toxicity was nitrate < urea < ammonium nitrate < ammonium.

Molybdenum as ammonium molybdate was very significantly more toxic than as sodium molybdate to growth in the nitrate series, and to a less, though significant, degree in the ammonium nitrate series, but no significant difference

in toxicity in the ammonium and urea series occurred between these two molybdenum sources (see Plate 3, Fig. 1). This result confirmed the observations previously made in Experiments 4 and 5. It is apparent that the presence of ammonium (NH_4^+) ions markedly increases the toxicity of molybdenum to flax. In the

TABLE 7
MEAN DRY WEIGHT PER PLANT (G.) AFTER FIVE WEEKS' GROWTH IN NUTRIENT SOLUTIONS. EXPERIMENT 6

Treatment†	Nitrogen Source							
	NO_3		NH_4NO_3		NH_4		Urea	
	Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots
Control	0.1722	0.0928	0.2713	0.1015	0.0899	0.0454	0.2113	0.1136
Mn 50 p.p.m.	0.0831	0.0561	0.2741	0.1210	0.1146	0.0517	0.1923	0.0986
Mo(NH_4) 10 p.p.m.	0.1391	0.0805	0.1495	0.0769	0.0463	0.0293	0.1471	0.0807
Mo(NH_4) 20 p.p.m.	0.0304	0.0232	0.0655	0.0501	0.0101	0.0100	0.0878	0.0619
Mo(Na) 10 p.p.m.	0.2130	0.1054	0.1698	0.0844	0.0368	0.0280	0.1614	0.0867
Mo(Na) 20 p.p.m.	0.1425	0.0844	0.1097	0.0764	0.0118	0.0117	0.1015	0.0714
Mn 50 p.p.m. + Mo(NH_4) 20 p.p.m.	0.0428	0.0361	0.0992	0.0725	0.0222	0.0174	0.0823	0.0569
Mn 50 p.p.m. + Mo(Na) 20 p.p.m.	0.0822	0.0624	0.0916	0.0688	0.0130	0.0117	0.0625	0.0437
Mn 50 p.p.m. + Mo(Na) 20 p.p.m. + NH_4 ($\equiv$ Mo (NH_4) 20 p.p.m.)	0.0927	0.0689						
							Tops	Roots
Least differences for significance between two means					5% Level		0.0270	0.0129
					1% Level		0.0357	0.0170
Significant differences required for testing interactions					5% Level		0.0382	0.0182
					1% Level		0.0505	0.0240

† See footnotes to Table 4.

absence of such ions (i.e. sodium molybdate added to the nitrate solution) a concentration of 10 p.p.m. of molybdenum in the nutrient solution actually produced a highly significant increase in top growth and an increase in root growth which was just not significant at the 5 per cent. level.

The results in Table 8 show that the manganese concentration was much greater in the plants grown in the presence of nitrate than in plants receiving

ammonium nitrate, ammonium, or urea as the nitrogen source. A comparison of Tables 6, 7, and 8 shows that manganese concentration alone does not determine whether the plants manifest necrotic leaf symptoms or any reduction in growth. Thus in the ammonium nitrate and ammonium series, the marked increase in manganese contents compared with the control, resulting from the

TABLE 8
MANGANESE AND MOLYBDENUM CONTENTS (P.P.M.) OF AERIAL PARTS OF PLANTS
ON DRY BASIS. EXPERIMENT 6

Treatment†	Nitrogen Source							
	NO ₃		NH ₄ NO ₃		NH ₄		Urea	
	Mn	Mo	Mn	Mo	Mn	Mo	Mn	Mo
Control	134	2	28	1	35	3	58	3
Mn 50 p.p.m.	3250	— ‡	1000	4	1120	9	1510	4
Mo(NH ₄) 10 p.p.m.	72	800	31	420	43	860	41	463
Mo(NH ₄) 20 p.p.m.	77	1525	30	710	30	1406	32	1125
Mo(Na) 10 p.p.m.	90	400	25	360	8	800	35	720
Mo(Na) 20 p.p.m.	90	970	35	720	25	1322	49	1520
Mn 50 p.p.m. + Mo (NH ₄) 20 p.p.m.	2980	1792	1750	966	930	1140	1460	1400
Mn 50 p.p.m. + Mo (Na) 20 p.p.m.	2700	1432	1680	1220	1030	1488	1950	1855
Mn 50 p.p.m. + Mo (Na) 20 p.p.m. + NH ₄ (≡ Mo (NH ₄) 20 p.p.m.)	2130	1138						

† See footnotes to Table 4.

‡ Sample lost. Plants 5 weeks old.

addition of an excess of this element to the nutrient solution, was not harmful to growth. Leaf necrotic symptoms of comparable severity occurred only in the plants receiving manganese alone in the nitrate and urea series. The manganese concentration in the latter plants was 1510 p.p.m. — less than half that in the nitrate plants. Also, this concentration of 1510 p.p.m. in the urea plants was less than that of other plants in the experiment not showing manganese-induced leaf necrosis. The suppression of such symptoms in these instances was evidently due to the concomitant high molybdenum concentration in the plants concerned.

The marked golden-yellow chlorosis which occurred in the plants grown in the solutions containing excesses of manganese and molybdenum was due

to some interaction resulting from the high concentrations of these two elements in the plant. However, no golden-yellow interaction occurred in plants containing high concentrations of manganese and molybdenum in the ammonium series.

In the presence of nitrate, plants receiving ammonium molybdate had a much higher molybdenum concentration than plants receiving the same amount of molybdenum as sodium molybdate. In the presence of the other nitrogen sources these two forms of molybdenum had the same effects on the molybdenum content of the plants. It is of importance to record that, in the nitrate series, a concentration of 400 p.p.m. of molybdenum in plants receiving sodium molybdate was accompanied by a significant increase in growth. Where ammonium (NH_4^+) ions were present in the nutrient solution a similar concentration of molybdenum in the plant was accompanied by a significant reduction in dry weight.

(g) Experiment 7

A further experiment was made to obtain additional data on the relative effects on plant growth of applications of manganese or molybdenum (as sodium molybdate) to nutrient solutions containing nitrate or ammonium respectively as the source of nitrogen. Each treatment was replicated 15 times. Six replicates were harvested after three weeks' growth and the remaining nine replicates after five weeks. The dry weights of the plants after these two periods are presented in Table 9.

Manganese-induced iron-deficiency chlorosis and lower leaf necrosis occurred only in the nitrate series. No chlorotic or necrotic symptoms occurred in any of the other plants in the experiment. In this experiment also, highly significant and opposite interactions occurred between nitrogen source (nitrate or ammonium) on the one hand and high levels of manganese or molybdenum on the other.

A statistical analysis of the combined data from the two water culture Experiments 6 and 7 is presented in Table 10. It was found that the effect of nitrogen source was greater after five weeks' growth than after three weeks, and also that the effect was greater in Experiment 7 than Experiment 6. Apart from this effect, interactions with experiments were not significant, thus enabling a direct comparison of means throughout the three sets of data.

In addition to confirming the significant interactions between nitrogen source and manganese or molybdenum, the important feature revealed by this analysis is that the increased growth of both tops and roots which resulted from the addition of 50 p.p.m. of manganese to the ammonium solution was highly significant.

IV. DISCUSSION

The ammonium and nitrate nutrition of plants have been the subject of considerable attention by various workers. Arnon (1937) noted important differences in the nutrition of barley plants grown in solutions supplied with either ammonium or nitrate as the sole source of nitrogen. Growth of the ammonium

plants was affected favourably and extensively by forced aeration or by the addition of small amounts of certain trace elements, including molybdenum and manganese. In the present experiments, growth of the flax plants in the ammonium solution was inferior to that in the other nitrogen sources. Best growth was obtained with ammonium nitrate. With peas, Mulder (1948a) demonstrated that the poor growth obtained with ammonium at relatively high

TABLE 9

DRY WEIGHTS PER PLANT (G.) AFTER THREE AND FIVE WEEKS' GROWTH IN NUTRIENT SOLUTIONS. EXPERIMENT 7, MARCH-APRIL 1950

Treatment	After 3 Weeks*				After 5 Weeks†			
	NO ₃		NH ₄		NO ₃		NH ₄	
	Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots
Control	0.0337	0.0181	0.0219	0.0113	0.1582	0.0812	0.0509	0.0278
Mn 50 p.p.m.	0.0154	0.0116	0.0247	0.0134	0.0571	0.0402	0.0583	0.0326
Mo(Na) 10 p.p.m.	0.0329	0.0187	0.0100	0.0076	0.1483	0.0836	0.0184	0.0165

Statistical Analysis

Table of Means ($\log x \times 10^4$)

	After 3 Weeks				After 5 Weeks			
	NO ₃		NH ₄		NO ₃		NH ₄	
	Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots
Control	2.522	2.242	2.332	2.038	3.180	2.894	2.704	2.437
Mn 50 p.p.m.	2.182	2.064	2.390	2.126	2.756	2.603	2.764	2.509
Mo(Na) 10 p.p.m.	2.510	2.267	1.989	1.870	3.165	2.919	2.260	2.211

	After 3 Weeks		After 5 Weeks	
	5% Level	1% Level	5% Level	1% Level
Significant differences between any two means	0.101	0.134	0.074	0.098
Interaction effect	0.143	0.190	0.105	0.139

* Six replicates.

† Nine replicates. Solutions renewed after three weeks.

hydrogen ion concentrations was due to injury by ammonium, which accumulated owing to an inadequate assimilation of this compound in the leaf tissues. An extremely high ammonium concentration was also found by Evans and Weeks (1948) in tobacco plants grown in an ammonium solution.

When plants were grown in a solution containing ammonium salts as the sole source of nitrogen, Jones and Shive (1921) and Arnon (1937) observed that the reaction of the culture medium rapidly became more acid, whereas with a nitrate solution, the reaction tended to become more alkaline. These findings have been confirmed in the present experiments.

It has been further shown (Arnon 1939; Friedrichsen 1944; Sideris and Young 1949) that nitrate plants have a greater absorptive power for manganese than plants grown in an ammonium solution. This result is confirmed by the results presented in the present paper. Previous work by Willis and Piland (1936) who grew cotton plants in solutions containing nitrogen in the form of nitrate or ammonium respectively, showed that the addition of manganese, zinc, or copper during early growth caused iron-deficiency chlorosis in both types of solution. However, in a second experiment in which the addition of the heavy metals was delayed, the chlorosis occurred only in the plants growing in the nitrate solution. This latter result with respect to manganese is supported by the experiments described above.

TABLE 10
STATISTICAL ANALYSIS OF COMBINED DATA FROM EXPERIMENTS 6 AND 7,
BASED ON LOG ($\times \times 10^4$)

	Tops		Roots	
	NO ₃	NH ₄	NO ₃	NH ₄
<i>Experiment</i>				
No. 6-5 weeks	3.158	2.855	2.911	2.603
No. 7-3 weeks	2.405	2.237	2.191	2.011
No. 7-5 weeks	3.034	2.576	2.805	2.386
<i>Treatment</i>				
Control	2.995	2.654	2.716	2.372
Mn 50 p.p.m.	2.624	2.724	2.477	2.445
Mo(Na) 10 p.p.m.	3.009	2.253	2.749	2.166
			5% Level	1% Level
Significant difference between any two means			0.048	0.064
Interaction effect			0.068	0.090

These experiments have also demonstrated that a concentration of manganese (50 p.p.m.) that was very toxic to growth in a nitrate solution produced a highly significant increase in growth in an ammonium solution even though with this latter nitrogen source the manganese concentration of the plants was very markedly increased when compared with the controls, although not as much as in the nitrate plants. This result gives support to the hypothesis suggested by Hewitt, Jones, and Williams (1949) that manganese is not required for nitrate reduction, but may be required at a later stage in nitrogen metabolism. In the presence of urea, which is absorbed by the plant unchanged (McKee 1949), the same concentration of manganese (50 p.p.m.) had a toxic effect on the plants. The severity of the induced chlorosis and necrosis was comparable with that of the nitrate plants, even though the manganese concentration in the former plants was only half that of the nitrate plants. It would appear that manganese is at least not required for the metabolism of urea to the same degree as it is for ammonium.

In nutrient solutions containing normal amounts of heavy metals, the form of nitrogen supplied to the plant has an effect on the utilization of iron. Leukel and Franch (1941) found that bahia grass utilized iron better when grown in an ammonium solution than in a nitrate solution, while Asana (1945) obtained inconclusive evidence that less iron was required by rice plants grown in an ammonium solution than in a nitrate solution. Previously Jones and Shive (1921) and Kapp (1934) had demonstrated, for wheat and rice respectively, that the toxic effect of iron in the solution was greater in the presence of ammonium than of nitrate. Similarly Sideris and Young (1946, 1949), in work with pineapples, showed that nitrate plants contained more total iron than the ammonium plants. However, there was an important difference in the distribution of the iron in the plant, in that a much greater percentage was localized in the roots of the nitrate plants compared with the ammonium plants. In tobacco however, Evans and Weeks (1948) found a higher iron content in plants grown in an ammonium culture than in a nitrate culture.

These reported effects of nitrogen source on the iron utilization of the plant have an important bearing on the effects of applications of heavy metals (including manganese) and of molybdenum. It has been reported by many workers, whose results have been largely summarized by Millikan (1947) and Hewitt (1948), that an excess of manganese, zinc, copper, cobalt, nickel, chromium, or lead in the nutrient substrate will induce iron-deficiency chlorosis in plants.

The results presented in this paper show that, with manganese at least, this effect of an excess of a heavy metal is related to the nitrate and urea nutrition of the plant, but not with ammonium nutrition. Attention has been directed by Arnon (1937) to computations by Myerhof, which indicate that nitrate is a very effective oxidizing agent in the plant. In the presence of a catalyst, such as an excess of manganese, it is possible that nitrate oxidizes the physiologically available ferrous iron to the physiologically unavailable ferric state, thus producing iron-deficiency symptoms in the plant. Sideris and Young (1949) suggest that the development of manganese-induced iron-deficiency chlorosis is due to the biochemical antagonism of homologous substances, whereby manganese is substituted for iron in protoporphyrin 9, the chlorophyll precursor, thereby inactivating the latter for subsequent conversion to chlorophyll. Tests with 1 per cent. diphenylamine in concentrated sulphuric acid have confirmed that plants supplied with nitrate and showing manganese-induced iron-deficiency chlorosis and lower leaf necrosis have a higher content of nitrate or other oxidizing materials than normal. However, no nitrate was detected in plants supplied with urea in which manganese-induced iron-deficiency chlorosis also occurred. Bennett (1945) has found that iron deficiency in plants is associated with a marked increase in soluble nitrogen in the tissues, although total nitrogen may be the same as that of normal plants. It is of interest to note here that manganese-deficient plants give a marked reaction to the diphenylamine test (Leeper 1941).

In addition to the effect on the severity of manganese-induced iron-deficiency chlorosis, and on growth, it is important to note that nitrogen source had a similar effect on manganese-induced lower leaf necrosis which is unrelated to the iron status of the plant (Millikan 1947; Hewitt 1948), i.e. manganese-induced lower leaf necrosis occurred in plants grown in nitrate and urea nutrient solutions, but was absent in those grown in ammonium- or nitrogen-deficient solutions.

With molybdenum, the evidence now accumulating in the literature indicates that an essential function of this element in plant life is that of catalysing the reduction of nitrate to ammonia. This work has been reviewed by Hewitt, Jones, and Nicholas (1947), Mulder (1948), Wilson and Waring (1948), and Stout and Meagher (1948). It has been demonstrated that, in molybdenum-deficient plants, there is a marked accumulation of nitrate, but a reduction in protein nitrogen and soluble organic nitrogen compounds. This high nitrate content of molybdenum-deficient plants is rapidly reduced following small applications of molybdenum to the substrate (Wilson and Waring 1948; Stout and Meagher 1948; Wilson 1949).

Mulder (1948) has further shown that tomato plants supplied with ammonium nitrogen, but no molybdenum, contained even higher amounts of organic nitrogen in the leaves and stems than plants supplied with nitrate plus molybdenum. He thus demonstrated that the molybdenum requirement of the plants was less in an ammonium solution than a nitrate solution. Previously Steinberg (1937, 1939) had come to the same conclusion in his work on the molybdenum requirement of *Aspergillus niger*. However, Vanselow and Datta (1949) found that deficiency symptoms developed in citrus in the absence of molybdenum, even when the plants were supplied with adequate nitrogen in the form of ammonium salts. The above findings with respect to the effect of nitrogen source on the molybdenum requirement of plants are supported by the results of water culture experiments presented in this paper.

It is shown that, in the absence of ammonium (NH_4^+) ions, the molybdenum tolerance of the flax plant may be surprisingly high, in view of the very small amount (0.01 p.p.m.) shown to be required for normal growth of plants (Arnon and Stout 1939). In the presence of nitrate only, a concentration of 400 p.p.m. of molybdenum in the plants was actually accompanied by a significant increase in growth in one experiment, whereas when ammonium (NH_4^+) ions were present in the nutrient solution a similar concentration of molybdenum in the plants was accompanied by a significant reduction in growth. Stout and Meagher (1948) have observed that there is a phosphate-molybdenum interrelationship in the plant. Thus the amounts of molybdenum translocated from the roots to the upper parts of the plant are strongly influenced by phosphate concentration. This interrelationship may be important in consideration of the present experiments. It has been stated, for example, by Dikuser (1934) that the phosphate content of plants is greater in the presence of ammonium than of nitrate. Evans and Weeks (1948) also found a higher phosphorus content in tobacco plants grown in ammonium cultures than in nitrate cultures.

These relationships in the plant between nitrogen source on the one hand and the manganese or molybdenum requirements on the other may provide an explanation for the manganese-molybdenum antagonism (i.e. the reduction in the severity of manganese-induced iron-deficiency chlorosis and lower leaf necrosis in the presence of excess molybdenum) reported by Millikan (1947, 1948, 1949a) and observed in the above experiments. Thus the nitrate which accumulates in the presence of excess manganese may oxidize ferrous iron to the ferric state, thereby inducing symptoms of iron deficiency, unless adequate molybdenum is available to ensure its reduction to organic nitrogen. There is evidence, however, that the presence of some ammonium ions facilitates the occurrence of this antagonistic phenomenon between manganese and molybdenum. Diphenylamine tests on plants grown in nitrate solutions in these experiments have confirmed that the existence of manganese-molybdenum antagonism is associated in this instance with the disappearance of nitrate accumulations in the plant.

However, further work on this antagonism is required. Thus, with urea as the source of nitrogen, excess manganese induced severe lower leaf necrosis in the plants, but when molybdenum was present the same excess of manganese caused no necrosis, even though the manganese concentrations in the two sets of plants were similar. In neither of these instances was nitrate detected in the plants.

It has also been shown that combined excesses of manganese and molybdenum induced a golden-yellow chlorosis of the leaves of flax in the presence of nitrate and urea, but not with ammonium. This symptom appears to be identical with that of severe molybdenum toxicity (Warington 1937, 1946; Millikan 1949b). It is not a symptom of iron deficiency and is not prevented by adding extra iron to the solution. All the factors influencing the occurrence of this golden-yellow interaction between manganese and molybdenum in the plant are not known. In some experiments conducted by the author the plants grown in the presence of a combined excess of these two elements have become golden-yellow in colour within three to four days, whereas in other experiments this same treatment has not induced the golden-yellow colour in the plants for several weeks. An increase in the molybdenum level in the solution may also reduce this golden-yellow chlorosis. In this regard Brenchley (1948) has observed that in certain soils this characteristic golden colour of the leaves of flax produced by applications of sodium molybdate was intensified by concurrent applications of manganese sulphate. She concludes, from the variable results obtained, that further investigation is required to elucidate the factors which determine the relative toxicity of molybdenum to plant growth. On the other hand, Hewitt (1949) in tests with sugar beet in sand cultures, has found that the addition of extra molybdenum to the culture induced a striking increase in the severity of manganese chlorosis.

The relative concentrations of amino acids in cauliflowers have been shown by Hewitt, Jones, and Williams (1949) to be dependent on the interaction of manganese and molybdenum. An increase in molybdenum level, particularly

under conditions of manganese deficiency, was paralleled by an increase in amino acid concentration, whereas an increase in manganese level, particularly in the presence of a high level of molybdenum, was associated with a reduction in amino acid concentration.

It has been shown by Millikan (1948, 1949*a*) that flax plants affected with lower leaf scorch, which respond to small applications of molybdenum under field conditions in Victoria, actually have a higher manganese concentration, and a higher Mn/Mo ratio than normal. Unpublished data of the author have also demonstrated that affected plants give a much more marked reaction to the diphenylamine test than normal plants, indicating an accumulation of nitrate in the former. Similarly Parbery (1937) found more manganese in cauliflowers grown in soil liable to "whiptail" than in plants grown in "healthy" soil. Chemical data obtained by Hewitt, Jones, and Nicholas (1947) also indicate that molybdenum deficiency symptoms of "whiptail" may possibly be induced by excess of manganese. Bean "scald," which is also associated with an excess of manganese in the affected plants when grown in highly acid soils high in available manganese (Parbery 1943), has been recently shown to be prevented by molybdenum (Wilson 1949). Cauliflower plants affected by "whiptail" (Hewitt, Jones, and Nicholas 1947; Wilson and Waring 1948) and bean plants affected by "scald" (Wilson 1949) are also high in nitrate.

Experience in Victoria has shown that whereas lower leaf scorch of flax may occur in the first crop grown on highly acid, newly ploughed, clover-grass lea ground, a second crop on the same area in the next season may be quite free of the disorder (Millikan 1948, 1949*a*). From the results reported in this paper it appears likely that a factor conducive to the disorder in the first crop would be a high nitrate content in the clover-lea ground, combined with the observed high manganese content. A similar explanation probably can be given for the reported effects of applications of sulphate of ammonia in increasing manganese toxicity or molybdenum deficiency symptoms in plants, the literature on which has been reviewed in part by Millikan (1948, 1949*a*). This relationship between sulphate of ammonia applications and molybdenum response is no doubt largely attributable to the rapid conversion of sulphate of ammonia to nitrate in the soil and its absorption by the plant as such. Recently Anderson and Spencer (1949) have reported that, in their experiments with non-legumes, responses to applications of molybdenum were obtained only by treatment with manganese sulphate in addition to sulphate of ammonia or nitric acid.

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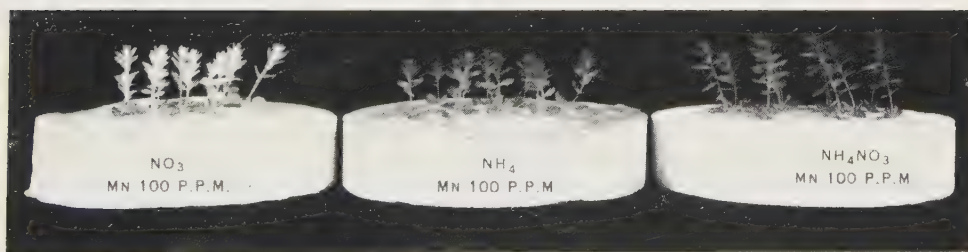
EXPLANATION OF PLATES 1-3

PLATE 1

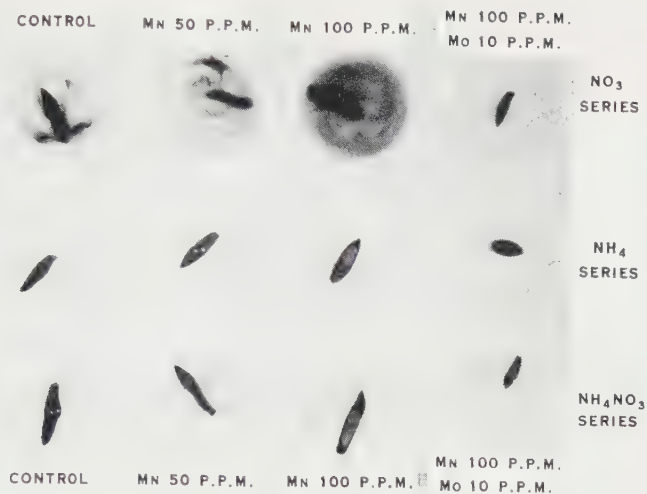
- Fig. 1.—Lower leaf necrosis in flax grown in a nitrate nutrient solution containing an excess of manganese.
- Fig. 2.—Portion of Experiment 2 showing the effects on flax of the addition of 100 p.p.m. of manganese to nutrient solutions supplied with nitrogen as nitrate, ammonium, or ammonium nitrate respectively. Plants grown in the nitrate solution showed manganese-induced iron-deficiency chlorosis and lower leaf necrosis, but these symptoms were absent in the plants grown in the ammonium or ammonium nitrate solutions.

PLATE 2

- Fig. 1.—Test for nitrate with 1 per cent. diphenylamine in concentrated sulphuric acid on leaves of flax plants from Experiment 2 grown in the presence of various nitrogen sources and with excess of manganese alone or with molybdenum. Manganese-induced iron-deficiency chlorosis occurred only in the plants in the nitrate solutions with excess manganese alone. The incidence of these toxicity symptoms was associated with an accumulation of nitrate as indicated by the development of the characteristic blue coloration with the diphenylamine reagent. The prevention of manganese toxicity symptoms in the nitrate series by the addition of molybdenum was associated with the disappearance of the excess nitrate.



MILLIKAN.—RELATION BETWEEN NITROGEN SOURCE AND THE EFFECTS ON FLAX OF AN EXCESS OF MANGANESE OR MOLYBDENUM IN THE NUTRIENT SOLUTION



MILLIKAN.—RELATION BETWEEN NITROGEN SOURCE AND THE EFFECTS ON FLAX OF AN EXCESS OF MANGANESE OR MOLYBDENUM IN THE NUTRIENT SOLUTION



Fig. 1



Fig. 2

MILLIKAN.—RELATION BETWEEN NITROGEN SOURCE AND THE EFFECTS ON FLAX OF AN EXCESS OF MANGANESE OR MOLYBDENUM IN THE NUTRIENT SOLUTION

Fig. 2.—Relative toxicity to flax of 20 p.p.m. of molybdenum supplied as ammonium molybdate and sodium molybdate respectively to a nitrate nutrient solution in Experiment 5.

PLATE 3

Fig. 1.—Representative pairs of plants from Experiment 6, showing the interaction between nitrogen source and the relative toxicity of 20 p.p.m. of molybdenum, supplied to each nitrogen series as ammonium molybdate (*A*) or sodium molybdate (*B*) respectively.

Fig. 2.—Golden-yellow chlorosis most evident in the tips of lower leaves, resulting from the addition of combined excesses of manganese and molybdenum to a nitrate nutrient solution.

STUDIES ON THE NITROGEN METABOLISM OF THE BARLEY PLANT (*HORDEUM SATIVUM*)

By H. S. McKEE*

[Manuscript received July 7, 1950]

Summary

In detached barley leaves held at constant temperature in the dark, there is an increase in "soluble" and a corresponding decrease in "solid" nitrogen. In mature leaves, glutamine accumulates in the early stages of starvation and later decreases concurrently with an increase in asparagine. In detached seedling leaves, the glutamine content remains low throughout, the sequence of events otherwise resembling that found with mature leaves. Changes in the nitrogenous fractions during autolysis, in sterile conditions, of barley leaf tissue differ greatly from those found with intact detached leaves. "Soluble" nitrogen increases, but neither amide nor ammonia accumulates.

In germinating seedlings, there is a considerable accumulation of asparagine. This accumulation is more marked in the dark than in the light, and in plants supplied with nitrogen as an ammonium salt than in those supplied with nitrate. Small amounts only of glutamine were found in seedlings. The relation of the results to our knowledge of nitrogen metabolism in plants is discussed.

I. INTRODUCTION

There is strong evidence (Yemm 1937) that much of the carbon dioxide produced in the respiration of detached barley leaves arises, especially in the later stages of starvation, from nitrogenous compounds. The present paper is mainly concerned with transformation of nitrogenous compounds in detached leaves and developing seedlings of barley. It is thought that a study of changes in nitrogenous substances, known from previous work to be of metabolic importance, may throw some light on the processes whereby products of protein catabolism provide substrates for respiration.

II. METHODS

All material used came from plants of *Hordeum sativum* (Plumage Archer variety) grown in conditions of known nitrogen supply. Mature leaves were taken from plants grown in glazed earthenware pots on the laboratory roof. Seedlings were grown in sand in glazed earthenware dishes, kept either in a warmed greenhouse, in a dark incubator at 21°C., or in a dark room at ordinary temperatures. All germinating seed was kept for the first 24 hours in an incubator at 25°C. The composition of the culture solution is given in Table 1; the three levels of nitrogen supply used are designated "2N," "N," and "2N/5."

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In the treatments with reduced supplies of nitrate, the supply of potassium was maintained by adding potassium chloride. In some cultures, the potassium nitrate was replaced by enough ammonium sulphate to supply an equal amount of nitrogen.

TABLE 1
COMPOSITION OF CULTURE SOLUTION (G./L.)

Calcium sulphate	0.374
Acid calcium phosphate	0.500
Magnesium sulphate	0.500
Sodium chloride	0.160
Ferric chloride	Trace
Potassium nitrate	2.72 for treatment "2N"
	1.36 for treatment "N"
	0.54 for treatment "2N/5"

III. EXPERIMENTAL CONDITIONS

Samples of detached leaves were held at 21°C. in darkened glass chambers immersed in a water-bath. In experiments where the respiratory output of carbon dioxide or uptake of oxygen was measured, these chambers remained unopened throughout; where samples were withdrawn for chemical analysis, the chambers were opened at each time of sampling. Air from outside the laboratory was drawn through the chambers after passing through cotton wool filters to remove solid particles and through baryta bubblers and soda-lime towers to remove atmospheric carbon dioxide. Finally it passed through a water bubbler, taking up enough moisture to prevent desiccation of the material. The carbon dioxide produced was absorbed by baryta in Pettenkofer tubes changed every three or six hours. In some experiments, the gas stream was switched from one to the next of a series of tubes by a clockwork device and in others the tubes were titrated and replaced at the end of each period. Mixed samples of leaves from the third, fourth, and fifth positions on the stem were used in all experiments. At the end of the experiments, the leaves were brown and sodden, but visible infection was rare, though no elaborate precautions were taken to ensure sterility.

IV. ANALYTICAL METHODS

(i) *Carbon Dioxide*.—The Pettenkofer tubes contained baryta of known strength (approximately N/10). The residual baryta was washed out and titrated with standard hydrochloric acid. Where respiratory quotients were determined, carbon dioxide and oxygen were both measured in the Haldane gas analysis apparatus.

(ii) *Extraction of Soluble Nitrogenous Compounds*.—Chibnall's ether-water method, as modified by Yemm (1937) was used. Material (5-10 g.) was cut finely, allowed to stand 15 minutes with 2 ml. ether and 15 ml. water, and pressed (2 tons/sq. in.) between tinned plates in a hydraulic press. Two

further lots of 15 ml. water were added and the sample pressed again 15 minutes after each addition of water. The juice from the three pressings was mixed, boiled for two minutes, filtered hot, cooled, and made to a known volume, aliquots being taken for the subsequent determinations. The residue on the filter paper was added to that from the press in some experiments and their total nitrogen ("solid nitrogen") determined.

(iii) *Estimation of "Solid Nitrogen."*—The residues from pressing and filtration were digested with concentrated sulphuric acid, potassium sulphate, and a little cupric sulphate, digestion being continued for six hours after the digest cleared, and the nitrogen content determined, using the Parnas-Wagner micro-Kjeldahl apparatus.

(iv) *Estimation of "Soluble Nitrogen."*—An aliquot of the filtrate was treated in the same way to determine the "soluble nitrogen."

(v) *Estimation of Ammonia.*—An aliquot of the filtrate was distilled in the micro-Kjeldahl apparatus at 45°C. under reduced pressure with a 10 per cent. aqueous suspension of magnesium oxide and the ammonia given off estimated as in the "solid nitrogen" determination.

(vi) *Estimation of Glutamine.*—The procedure of Chibnall and Westall (1932) was followed. An aliquot of the filtrate was hydrolysed for three hours in a boiling water-bath with an acetate buffer (pH 4) and the ammonia produced estimated as before. The extra ammonia, compared with that produced on distillation of the unhydrolysed extract with magnesium oxide, was taken as arising by hydrolysis of glutamine. Certain modifications of the method of Chibnall and Westall (1932) were recommended by Vickery *et al.* (1935); in particular, that the pH of the hydrolysing buffer should be raised from 4 to 6.5, as urea and allantoin can interfere at the lower pH. At pH 6.5 hydrolysis of urea is stated to be incomplete after 20 hours on a boiling water-bath. Eight extracts from leaves starved for periods up to 166 hours were hydrolysed (*a*) for 2 hours with a phthalate buffer at pH 6.3, (*b*) for 3 hours at pH 6.3, (*c*) for 3 hours at pH 4. Differences between the three sets of figures were negligible and it was therefore assumed that interfering substances were not present in significant amounts in the experimental material, though urea is known to occur in barley leaves in very small amounts (Fosse 1913; Klein and Tauböck 1931).

(vii) *Estimation of Asparagine.*—It was found, in accordance with the results of Yemm (1937), that 4 hours heating in a boiling water-bath of an asparagine solution made 2N with respect to sulphuric acid gave 98 per cent. hydrolysis and that further heating gave no increase on this figure. These conditions were therefore adopted. The buffer (pH 4) used for the glutamine hydrolysis gave 3.4 per cent. hydrolysis with asparagine; this causes no serious error in the glutamine estimations, as is shown by the very small amounts of glutamine found on some occasions in the presence of large amounts of asparagine.

(viii) *Estimation of Nitrate*.—The residue from the glutamine estimations (after distillation of the ammonia) was made more strongly alkaline by addition of 3 ml. of 30 per cent. sodium hydroxide to 20 ml. of solution, and Devarda's alloy (Sessions and Shive 1928) added to reduce nitrate to ammonia, which was estimated by distillation as usual, though serious difficulty was met through excessive frothing under reduced pressure. For this reason, nitrate determinations were made only when the results seemed likely to be of particular interest. The method gave excellent recoveries on pure nitrate solutions and it was also established that asparagine was not hydrolysed in the alkaline conditions used.

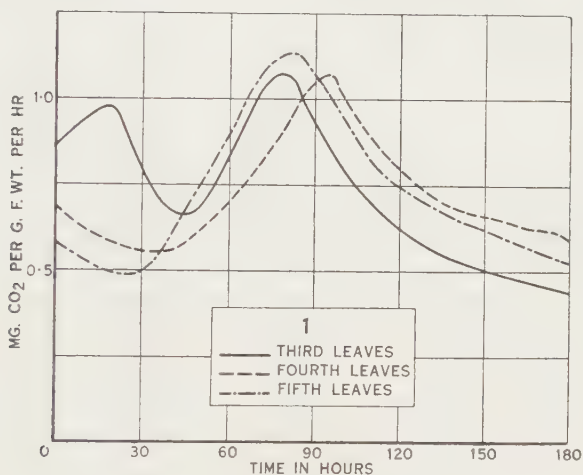


Fig. 1

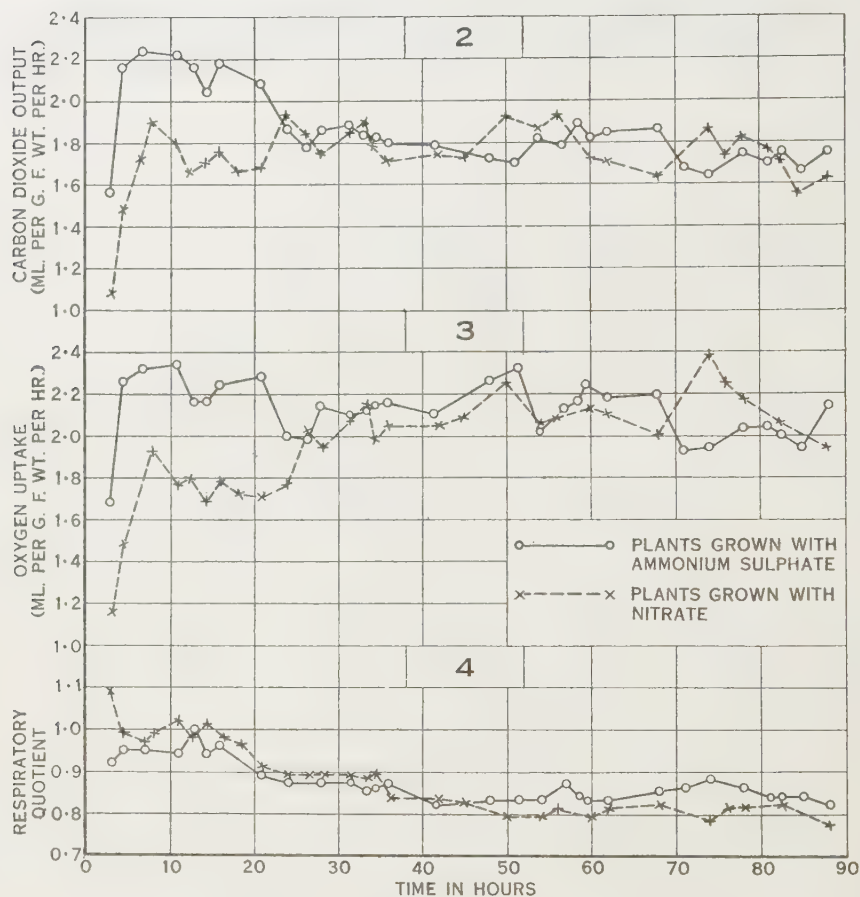
IV. EXPRESSION OF RESULTS

A single sample was used throughout an experiment for measurement of carbon dioxide output, but the chemical determinations require a new sub-sample for each occasion of sampling. The regularity of the changes in the various constituents found in most experiments indicates that the sub-samples used have been reasonably comparable with one another. For organs that have ceased to develop, such as detached leaves, the amount of each metabolite per unit fresh weight is recorded. This, however, may obscure metabolic changes in such rapidly developing material as seedlings, and for these the results are expressed in terms of both individuals and fresh weight. In the respiration experiments, the fresh weight used is that at picking and in the analytical work that at the time of sampling. Changes in weight during the experimental period are thus disregarded, but it is believed they are not great enough to distort seriously the general metabolic picture.

V. RESULTS

(a) Carbon Dioxide Output

Plotted against time, the carbon dioxide output of isolated darkened barley leaves, held at a constant temperature, gives a characteristic two-humped curve. Figure 1 shows a set of such curves for leaves from each of three positions on the stem. Similar data were collected for leaves from plants grown with other



Figs. 2-4

levels of nitrogen supply, but no consistent effect of nitrogen supply on the form or position of the respiration curve could be demonstrated. The respiratory behaviour of leaves from seedlings grown with nitrogen supplied as ammonium and nitrate was compared. Over the first 20 hours the former showed a higher carbon dioxide output and oxygen uptake, but later the curves came together. The "ammonium" leaves had a lower respiratory quotient for the first 35 hours, after which the curves crossed and the "ammonium" leaves had the higher quotient till the end of the experiment (Figs. 2, 3, and 4).

(b) Changes in Nitrogenous Constituents

(i) *Detached Mature Leaves*.—Six experiments were run with this type of material, three in one season and three in the next. The results all conform to the general pattern found for field-grown material by Yemm (1937). The qualitative course of nitrogen metabolism is similar in detached leaves from plants grown at the three levels of nitrogen supply, though, as might be expected, leaves from plants grown with the higher supplies of nitrogen form,

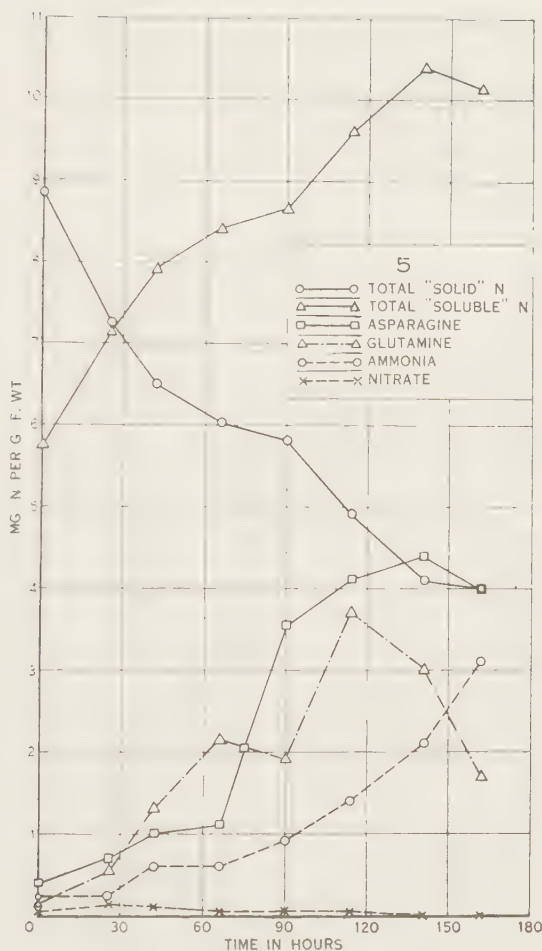


Fig. 5

during starvation, larger amounts of ammonia and the amides. Glutamine reaches a high concentration comparatively soon after the leaves are detached; it then decreases concurrently with an increase in the asparagine concentration. The latter also falls towards the end of the experiment, sometimes much more steeply than in the case illustrated. The ammonia concentration rises slowly in the earlier part of the experiment and becomes large towards its end. The

final decrease in asparagine content occurs after about 140 hours, the precise time varying from sample to sample, and coincides with the final collapse of the starved material (Fig. 5).

At this stage, the leaves are changing from yellow to brown and the content of soluble carbohydrate is probably very low (Yemm 1935). The figures for ammonia, high as they are towards the end of the experiments, do not represent all that is formed in the tissues of the detached leaves, for some escapes in gaseous form. In one respiration experiment with detached leaves from etiolated seedlings the gas stream from the respiration chamber was passed through a bubbler containing a known amount of dilute acid. In this, ammonia was identified using Nessler's reagent and estimated by back titration of the

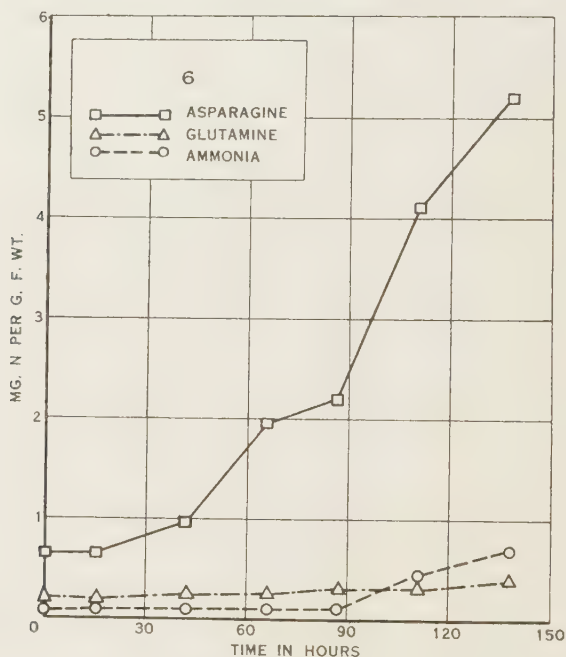


Fig. 6

acid to be equivalent to 0.35 mg. of ammonia per g. of original fresh weight, given off in 150 hours. Very small amounts of ammonia have been shown (Klein and Steiner 1928; Steiner and Löffler 1931) to be lost from leaves of many plants under normal conditions, but the losses in starvation are much greater. The results shown in Figure 5 may be taken as typical of the results with mature detached leaves. The increase in "soluble" and decrease in "solid" (presumably mainly protein) nitrogen are rapid and continuous from the beginning of the experiment; there is no indication of an initial lag in protein hydrolysis or of a sparing of protein by carbohydrate, such as were found with detached tobacco leaves by Vickery *et al.* (1933), with vine leaves by Deleano (1912), and with Kikuyu grass leaves by Wood, Cruickshank, and Kuchel

(1943). The amounts of nitrate found are small throughout, but there is an increase during the first 24 hours after the isolation of the leaves. This is a surprising finding, but similar results have been reported for tobacco (Vickery *et al.* 1933), tomato, and Swiss chard (*Beta vulgaris* var. *cicla*) leaves (McKee and Lobb 1938).



Fig. 7

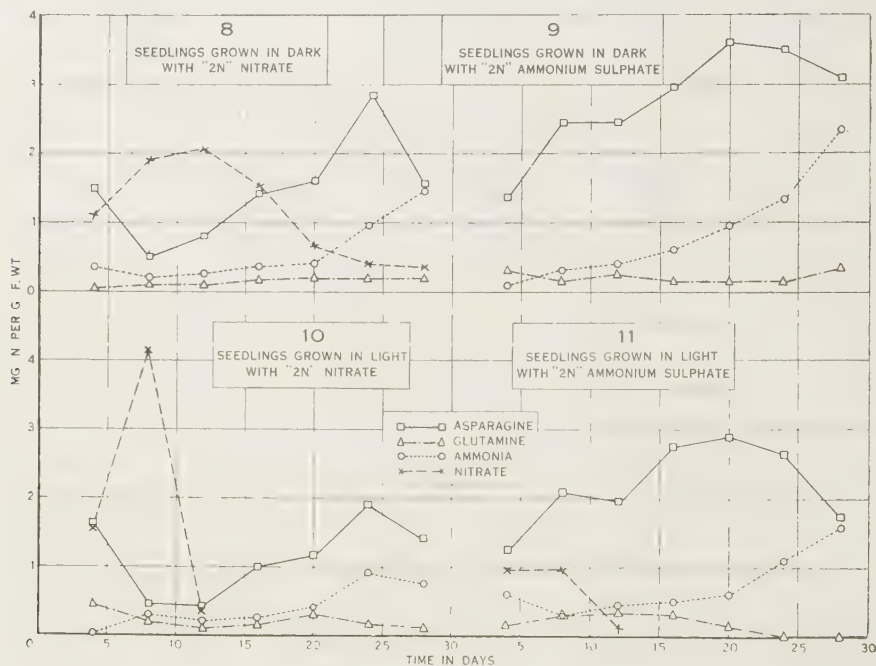
(ii) *Results with Detached Seedling Leaves.*—Five experiments were run with detached seedling leaves. The material included leaves from plants grown in the light and in the dark, and with nitrogen supplied as nitrate and as ammonium. The leaves from the plants supplied with ammonium had more asparagine initially than those given nitrate; their subsequent rate of asparagine accumulation and maximum asparagine concentration were also higher than in the "nitrate" leaves. Glutamine in both sets was present in low concentrations, but there was rather more in the "ammonium" leaves.

Leaves detached from seedlings accumulate much less glutamine than those from mature plants. The glutamine content also fails to show the early maximum found in starvation experiments with mature leaves. Typical results are shown in Figure 6.

(iii) *Autolysis Experiments.*—Two experiments were run, both using four-weeks-old seedling material grown in the light with "2N" nitrate supply. The material was cut finely and ground in a mortar. Samples (5 g.) were incubated at 21°C. with 50 ml. water and a little toluol in flasks fitted with cotton wool stoppers. In one experiment five analyses were made at intervals from 24 to 120 hours, and in the other four analyses from 120 to 287 hours. The results, shown in Figure 7, differ markedly from those with detached intact leaves.

There is a steady decrease in "solid" and a corresponding increase in "soluble" nitrogen, which does not appear to any important extent as ammonia or amide, but in some other form. After 287 hours, the ammonia content is still low and amides have almost disappeared.

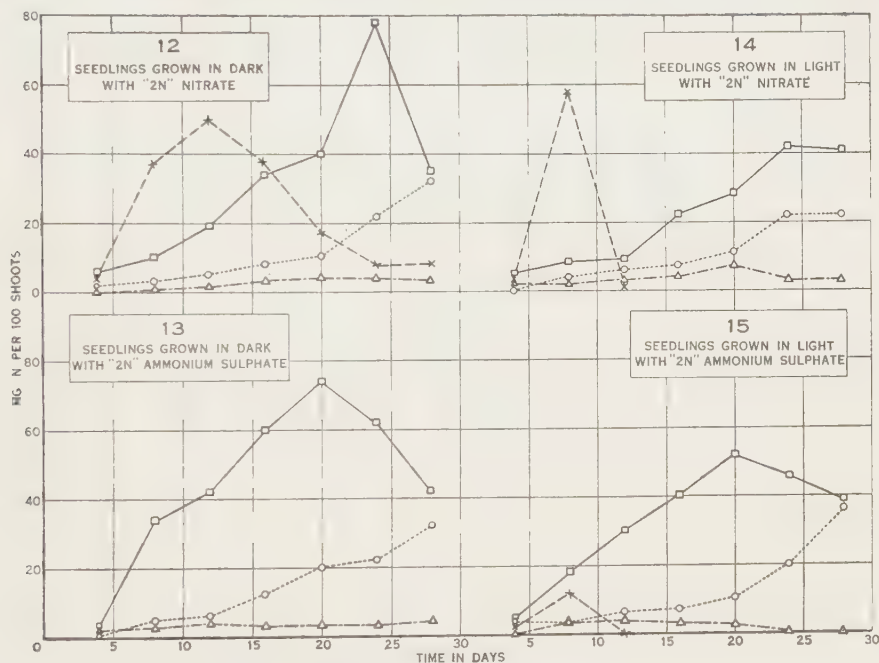
(iv) *Changes in Nitrogenous Constituents of Developing Seedlings.*—Four experiments were run, using seedlings grown with nitrate and ammonium (both "2N") in light and darkness. The results are presented both in terms of mg. N/g. fresh weight (Figs. 8, 9, 10, and 11) and of mg. N/100 shoots for the various constituents (Figs. 12, 13, 14, and 15). Over the first 20 days there is



Figs. 8-11

in all sets a great increase in the amount of asparagine per shoot. After 20 days, the asparagine content falls considerably in all sets except that grown with nitrate in light. In the other three sets, a decrease in the asparagine content is associated with a rise in that of ammonia, which rises steadily from the beginning in all sets except that grown with nitrate in light. Glutamine in all sets is present always in very small amounts compared with asparagine; it tends, in most cases, to decrease towards the end of the experiment. Both sets of etiolated seedlings reach a maximum weight between 16 and 20 days and subsequently lose weight. Both in the light and dark the "nitrate" seedlings are heavier than the "ammonium" seedlings (see Fig. 16). The nitrate content of seedlings supplied with nitrate and grown in the dark increases up to 12 days and then declines till at 28 days only comparatively small amounts are present. In the light, nitrate concentration is very high after eight days, but falls to a low value within three days.

A marked decrease in the concentrations of ammonia and asparagine occurs in the "nitrate" seedlings, both in the light and dark, between the fourth and eighth days. This is masked, when the results are calculated per shoot, by the rapid increase in bulk of the seedlings at this period. In the "ammonium" sets, asparagine and ammonia increase from the beginning even when calculated on the fresh weight basis. The finding of Vickery, Pucher, and Clarke (1936) that glutamine accumulates mainly in the root of sugar beet suggests that in any repetition of these experiments, it would be desirable to analyse the roots as well as the shoots of the seedlings, especially as Yemm (1949) has shown that barley roots assimilating nitrate or ammonium ions form glutamine rapidly.



Figs. 12-15

VI. DISCUSSION

It has long been held that the formation of amides in the plant is a response to the presence in the cell of dangerously high concentrations of ammonia. This view can be traced back to Boussingault (1864), and has more recently been strongly supported by Pryanishnikov (1924). In the experiments reported here, formation of ammonia in the tissues or its supply from outside led to the formation of large amounts of asparagine, both in intact seedlings and in leaves detached from seedlings and mature plants. Glutamine, however, shows, in the conditions of these experiments, no tendency to accumulate with rising ammonia concentration, and is never found in large amounts in seedling material. The relation between asparagine and glutamine may be expressed by the regression equation:

$$y = a + bx,$$

where y is the concentration of glutamine in mg. N per 100 g. fresh weight and x that of asparagine. Values of a and b for different types of material are shown in Table 2.

TABLE 2
VALUES OF a AND b IN BARLEY LEAVES

Material	No. of Observations	a	b	Standard Error of b	Significance of Difference of b from Zero
Detached mature leaves	38	0.73	0.43	0.06	Highly significant
Detached seedling leaves	17	0.19	0.05	0.02	Not significant
Intact seedlings	28	0.24	0.04	0.05	Not significant

There is, therefore, in mature but not in seedling material, a statistically demonstrable increase of glutamine content as that of asparagine rises.

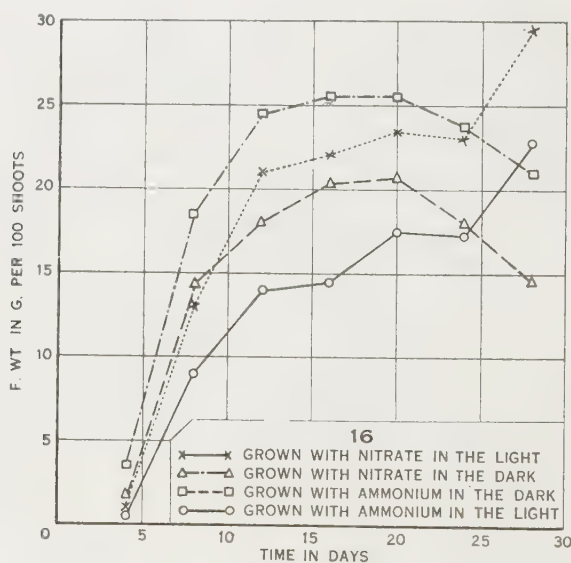


Fig. 16

In all experiments with young material, whether intact seedlings or young leaves, grown in the light or the dark, on nitrate or ammonium salts, the glutamine content is always much lower than that of asparagine. It is thus clear that the formation of ammonia in barley tissues or its introduction from outside the plant leads to accumulation of asparagine rather than of glutamine, and we must seek some other explanation for the accumulation of glutamine in the early stages of the starvation experiments with detached leaves. It may arise in part by hydrolysis of reserve protein. The leaf proteins of barley yield large amounts of glutamic acid and ammonia on acid hydrolysis (Yemm 1950) and it seems possible that part at least of this may represent glutaminy units in the

protein molecule, as demonstrated for gliadin by Damodaran, Jaaback, and Chibnall (1932). The key to the behaviour of glutamine may also involve the intermediate products of carbohydrate breakdown. Glutamine is closely related to glutamic acid and so to α -ketoglutaric acid, a component of the tricarboxylic acid cycle which plays a major part in the catabolism of carbohydrate (via pyruvic acid) and fat (via a two-carbon-atom compound whose exact nature is uncertain). The relations between asparagine, aspartate, and oxalacetate and between glutamine, glutamate, and α -ketoglutarate show the close connection of the cycle with some of the most important compounds in the intermediate nitrogenous metabolism of plants. The consequent interrelations are discussed elsewhere in some detail (McKee 1937, 1949), and have also been considered recently by Yemm (1949).

VII. ACKNOWLEDGMENTS

The experimental work described in this paper was carried out in the Botany Department, University of Oxford, during 1935 and 1936. The author wishes to thank Dr. W. O. James of that Department for his interest in the problem and Dr. E. W. Yemm, now of the University of Bristol, for advice on experimental techniques and for discussion of the interpretation of the results. He is indebted also to Professor J. G. Wood of the University of Adelaide and to Dr. R. N. Robertson of the Division of Food Preservation and Transport, C.S.I.R.O., for criticism of the manuscript, and to Mr. G. E. Ferris, Section of Mathematical Statistics, C.S.I.R.O., for calculating regression equations to illustrate the relations between asparagine and glutamine in different types of material.

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STUDIES IN THE METABOLISM OF PLANT CELLS

VIII. DEPENDENCE OF SALT ACCUMULATION AND SALT RESPIRATION UPON THE CYTOCHROME SYSTEM

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Summary

It has been established with the use of the enzyme inhibitor carbon monoxide that the cyanide-sensitive salt respiration in carrot root cells is mediated by the cytochrome-cytochrome oxidase system. The cyanide-stable ground respiration is not mediated by the cytochrome-cytochrome oxidase system.

The cyanide-sensitive process of ion accumulation in carrot root cells is reversibly inhibited by carbon monoxide in exactly the same way as is the salt respiration, and is thus also closely dependent on the activity of the cytochrome-cytochrome oxidase system.

This work thus establishes the validity of the previous assumptions that cyanide inhibition of these processes was due to inhibition of the cytochrome-cytochrome oxidase system.

I. INTRODUCTION

The process of salt accumulation by plant cells is generally accompanied by an enhanced respiration. Various workers (Lundegardh 1945; Machlis 1944; Milthorpe and Robertson 1948) have shown that, with cereal root cells in salt solution, respiratory poisons—cyanide, azide, malonate, and iodoacetate—inhibit both the salt accumulation and a part of the total respiration. It has further been shown that, in root cells of wheat, barley, carrot, and red beet, the salt respiration and the salt accumulation are completely inhibited by cyanide, while the respiration in water is not inhibited or partially inhibited (Lundegardh 1945; Robertson and Turner 1945; Robertson, Turner, and Wilkins 1947; Milthorpe and Robertson 1948). It has been assumed in the above papers that the cytochrome-cytochrome oxidase system mediates the cyanide-sensitive respiration of all the tissues investigated, and is also linked in some way to the process of salt accumulation. Lundegardh's theory of salt accumulation is based upon the assumption that such a linkage exists. This assumption has yet to be proved.

Marsh and Goddard (1939) have already shown, by the use of the inhibitor carbon monoxide, that cytochrome oxidase mediates a large part of the total respiration of carrot root cells immersed in a phosphate buffer solution.

In the present paper the authors report experiments planned to discover whether the enzyme system whose activity was shown to be essential for salt respiration and accumulation in carrot cells (Robertson and Turner 1945) is in fact the cytochrome system. Carbon monoxide has been used as a specific inhibitor.

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II. MATERIAL AND METHODS

These were very largely those described in earlier papers of this series. The tissue used was secondary parenchyma from carrot root (*Daucus carota* L.). In earlier experiments phloem tissue, and in the later experiments xylem, was used. The present work is thus linked with that done earlier on both these tissues. Two separate lots of carrots (var. Danvers Half-Long) were obtained from one farm, the leaves removed, and the roots stored in moist sand in the dark at 5°C. until required. The tissue, which remained quite turgid, was cut by microtome into discs, 0.8 cm. diameter and either 1 mm. or 0.75 mm. in thickness, as stated below. (This difference in disc thickness appeared to have no significant effect.) The discs were well rinsed after cutting, and then aerated in distilled water until required. They were used for experiments at periods varying from 70 to 500 hours from cutting; only healthy turgid discs were used.

Respiration rates were determined at $25 \pm 0.05^\circ\text{C}.$, by standard Warburg technique. The oxygen uptake of 0.9-1.0 g. of discs was measured. Rates were expressed as cu. mm. O_2 per g. fresh weight of tissue per hour. Each set of discs was lightly blotted dry, weighed, and transferred to the vessels with 4 ml. of distilled water or of the required solution. Potassium nitrate and potassium chloride were the salts used in the experiments, since they are known to cause the same respiratory responses, and are accumulated at the same rate by the cells (Wilkins and Weeks, unpublished data). All concentrations quoted are those of the solution in contact with the carrot slices. Concentrations of 0.075M and 0.1M were used so that the concentration of the salt should not limit accumulation or respiration (Robertson and Wilkins 1948).

In experiments with potassium cyanide the potassium hydroxide in the centre wells was replaced by the appropriate KOH-KCN mixture (Krebs 1935). Since the respiration of tissue aerated for long periods was occasionally stimulated by the addition of KCN solution, freshly prepared cyanide solutions, containing an equal concentration of phosphate buffer (pH 6.2), were used to maintain the pH of the solution below 8, thus eliminating cyanide stimulations almost entirely. In absence of stimulation, no difference was noted in respiration rates in unbuffered aqueous KCN, in KCN solution buffered in this way, or in KCN buffered with KH_2PO_4 .

Salt accumulation was measured in replicate sets of tissue in small flasks immersed in the thermostat and attached to the Warburg shaker for aeration. Sets of approximately 2.3 g. of discs (fresh wt.) were used in 10 ml. of salt solution—i.e. the same tissue-solution ratio as for respiration measurements. Accumulation was followed by withdrawing samples at intervals from the external solution and measuring the conductivities in conductivity pipettes and returning the samples before the next measurement. The advantages of the conductivity technique have been discussed previously (Robertson 1941).

Carbon monoxide was prepared by dehydrating formic acid with warm, concentrated sulphuric acid, washing the gas twice through sodium hydroxide solution, and collecting it in jars. From these jars a known volume of CO was

withdrawn and replaced by pure O_2 to give the required gas mixture. Nitrogen-oxygen mixtures were obtained commercially or prepared from pure N_2 and O_2 by displacement methods. Most of the gas mixtures were analysed after completion of each experiment. The evacuation procedure of Umbreit, Burris, and Stauffer (1945) was used to supply mixtures of CO and O_2 to both the Warburg vessels and the accumulation flasks. To avoid vessel breakages, N_2 - O_2 mixtures were sometimes supplied to the Warburg flasks by passing a rapid gas stream through the tap and flask and out of the loosened side-arm stopper for approximately 8-9 minutes while shaking the flask. The side-arm was then closed before stopping the gas stream. No difference was noted in the results whether this or the evacuation technique was used. The evacuation procedure *per se* was shown not to alter the respiration rate of the discs.

Levy and Schade (1948) report a small oxidation of CO proceeding on the alkali-soaked filter papers in Warburg vessels. This was not found to occur here at 25°C.

Both Warburg vessels and accumulation flasks were shaken at 117-124 strokes/min. (3 cm. excursion); increase in shaking rate from 100 to 120 strokes/min. caused no increase in respiratory rate in an atmosphere of 5 per cent. O_2 , the lowest O_2 concentration used. After each experiment the solutions bathing the discs were examined, and the discs tested for turgidity.

Illumination for the reversal of CO inhibition was supplied as below. Light meter readings given have been corrected for colour sensitivity.

"Full" light: a 400 W. mercury vapour lamp giving 350 foot-candles at the bottom of the vessels.

"Blue" light: by passing the mercury light through a cuprammonium sulphate filter solution, giving 180 foot-candles at the bottom of the vessels. (Composition: Hg emission bands at 436, 408, and 405 $m\mu$ only.)

"Red" light: a 1000 W. tungsten filament light with an aqueous safranin filter, giving 117 foot-candles = 65 per cent. intensity of "blue" light. (Composition: a broad region, 590-700 $m\mu$ approximately, maximum at 650 $m\mu$.)

Darkness was obtained by wrapping vessels in black cloth.

In each experiment, respiration and accumulation rates were first established at steady levels or as steady drifts in air, before applying any treatment. Respiration rates in air and in various treatments were obtained by extrapolating the steady level or drift, once attained, backward or forward to the moment of applying the treatment, to give initial or final rates, respectively. This is the only way in which a true comparison of rates under two different conditions may be made on the one set of tissue, in which the respiration rate is drifting. Rates in CO- O_2 gas mixtures in the dark are comparable with control rates in N_2 - O_2 only at the moment of supplying the gases, since there is a different rate of fall of O_2 content, due to respiration, in the two mixtures.

Results are expressed as percentages of the appropriate control rates; such percentage values are, of course, comparable only within any one experiment. In calculating these percentages, the small differences in the initial rates in air

were allowed for by correcting to the mean value. Each experiment was done at least twice; each treatment within an experiment was duplicated wherever possible.

III. RESULTS

(a) *Phloem and Xylem Parenchyma*

The behaviour of the phloem and xylem tissues in the present work confirmed Turner and Hanly's (1949) observation that the respiration of the tissues is qualitatively similar. They also found the xylem to respire at a lower rate than the phloem on a fresh weight basis, but at the same rate on a protein nitrogen basis.

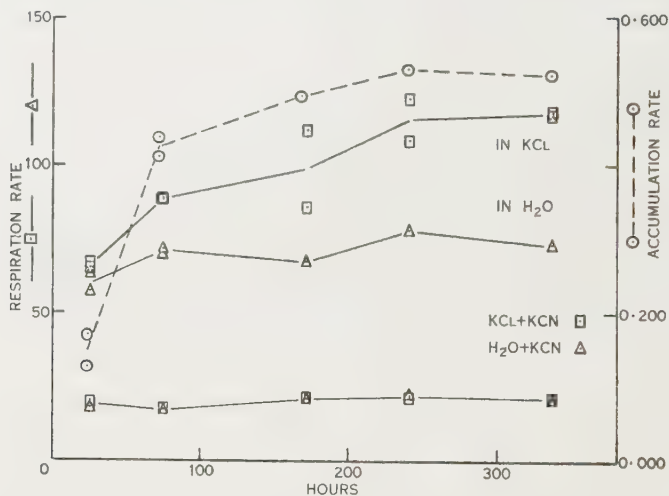


Fig. 1.—Changes with time in rates of accumulation and of respiration of tissue immersed in water, in KCl solution, and in each of these together with KCN. Each point is obtained from separate experiments on replicate sets of xylem parenchyma from a single carrot.

Tissue cut at 0 hours. Respiration rates, cu. mm. O_2 /g. f.wt./hr.; accumulation rates, g. mol. KCl/g. f.wt./hr. $\times 10^5$. Discs 0.75 mm. thick; KCl, 0.1M; KCN, 0.001M.

(b) *Respiratory Quotients*

A few experiments to determine the R.Q. of carrot discs respiring in water and in salt solutions yielded results confirming those of Turner and Hanly (1949). The R.Q.'s in KCl and in KNO_3 solutions and in water were not significantly different from unity. If this tissue were similar in behaviour to the barley roots used by Ulrich (1941), this would indicate that these anions and cations were being absorbed in approximately equal quantities.

(c) *Inhibition of Respiration by Cyanide*

The respiratory behaviour of the tissue used in this investigation is somewhat different from that in the earlier work (Robertson and Turner 1945) in

which the respiration of discs which had been aerated in water for more than 120 hours was unaffected by KCN. Cutting the discs always induces a cyanide-sensitive wound respiration; the persistence of this depends on the season in which the carrots are dug, their age, and, to a much smaller extent, the temperature during aeration.

It is seen from Figure 1 that the tissue used here showed, at all times from cutting, a water respiration partially sensitive to KCN, similar to that of the type B carrots described by Turner and Hanly (1949). The respiration in distilled water thus consists of two qualitatively different fractions, one stable towards inhibitors (e.g. KCN), the ground respiration; the other similar to the cyanide-sensitive salt respiration.

(d) Carbon Monoxide Inhibition of Respiration

Quantitative comparisons of respiration rate in CO-O_2 and $\text{N}_2\text{-O}_2$ mixtures are valid only where the same O_2 concentrations exist in both. With the available apparatus, difficulty was experienced in early experiments in obtaining the same O_2 concentration in both mixtures. Some of these results are useful qualitatively, however, to show the general effect of supplying the gases to tissue respiring under various conditions. The O_2 concentration in the $\text{N}_2\text{-O}_2$ control gas was always lower than that in the CO-O_2 mixture; thus CO inhibitions appeared smaller than they really were.

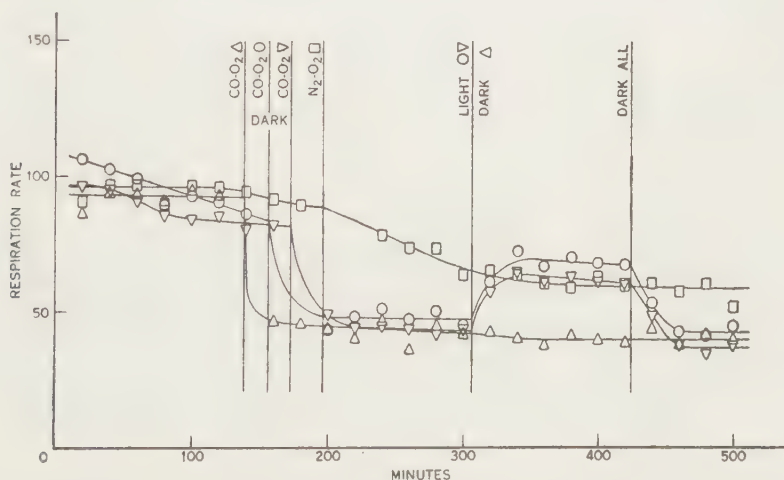


Fig. 2.—Effect of carbon monoxide on the respiration rate of phloem parenchyma in KNO_3 solution.

Respiration rate, cu. mm. O_2 /g. f.wt./hr. Discs 1 mm. thick; KNO_3 , 0.75M. Gases, 90% CO + 7.5% O_2 and 94% N_2 + 6% O_2 . Time from cutting, 500 hr.

Two of these experiments are shown in Figures 2 and 3. From Figure 2 it is evident that the total respiration of carrot discs in a salt solution is partially inhibited by CO, the effect exceeding greatly that of decreasing O_2 concentration alone. Strong illumination reverses the inhibition occurring in the dark.

Figure 3 shows that, with gas mixtures of higher O_2 concentration, it is possible to obtain CO-inhibition of the salt respiration, while the respiration in water remains unaffected. Figures 2 and 4 illustrate the similarity of the effect of CO upon the respiration of both xylem and phloem tissue. The extent of inhibition by CO was similar for tissue in both KCl and KNO_3 solution, as shown in Table 1.

TABLE 1
EFFECTS OF LOW OXYGEN CONCENTRATION AND OF CARBON MONOXIDE ON THE
RESPIRATION RATE OF XYLEM PARENCHYMA IN KCl AND IN KNO_3 SOLUTION
Discs 1 mm. thick; KCl and KNO_3 , 0.075M; gases, 93% CO + 7% O_2 , and 94.5% N_2 +
5.5% O_2 ; time from cutting, 238 hr.

	Respiration rate (cu. mm. O_2 /g. f. wt./hr.)							Percentages			
	Air	CO- O_2 Light Initial	CO- O_2 Dark Initial	N_2 - O_2 Light Initial	CO O_2 Light Final	N_2 - O_2 at Dark	CO- O_2 Dark Initial	CO- O_2 Light Final	CO- O_2 Dark Initial	CO- O_2 Light Initial	N_2 - O_2 Light Initial
KCl	77.5	—	39.6	—	—	—	—				
KCl	65.5	61.3	—	—	56.8	—	44.6	127.3	105.5	68.2	
KCl	76.0	—	—	58.1	—	53.3	—				
KNO_3	75.5	—	43.4	—	—	—	—				
KNO_3	63.0	55.5	—	—	50.4	—	40.7	123.8	90.8	71.0	
KNO_3	66.5	—	—	61.1	—	57.5	—				

From the experiments illustrated by Figure 4, in which the O_2 concentration was the same in both the gas mixtures, it was evident that the "ground" or cyanide-stable respiration was stable towards CO as well as to cyanide. As illustrated by the data in Table 2, the total respiration of tissue in water was always partially sensitive to CO-5 per cent. O_2 , and to KCN, indicating the persistence of a fraction of the respiration similar to the salt respiration. The salt respiration itself was in every case inhibited by CO.

The variation in response of the tissue towards O_2 concentrations lower than 21 per cent. depended on the discs from each carrot, and particularly on their respiration rate. In tissue with respiration rates below 60-70 μ l. O_2 /g. f. wt./hr., N_2 - O_2 mixtures with only 5.5 per cent. O_2 frequently caused no depression of respiration rate. The respiration of tissues which respired at higher rates in air was generally depressed in O_2 concentrations of 5-9 per cent., the slow fall in rate occurring gradually along a hyperbolic curve or straight line irrespective of the method used to supply gas mixtures to the tissue. This contrasted with the rapid depression to a steady rate generally occurring in CO- O_2 mixtures.

(e) Relative Affinities of Enzyme towards CO and O₂

Since CO competes with O₂ for the enzyme affected, its inhibiting action is always only partial, and the fraction of the total respiration mediated by the sensitive enzyme cannot be directly determined. Since the inhibitory action of KCN upon cyanide-sensitive enzymes is non-competitive, it can be made almost

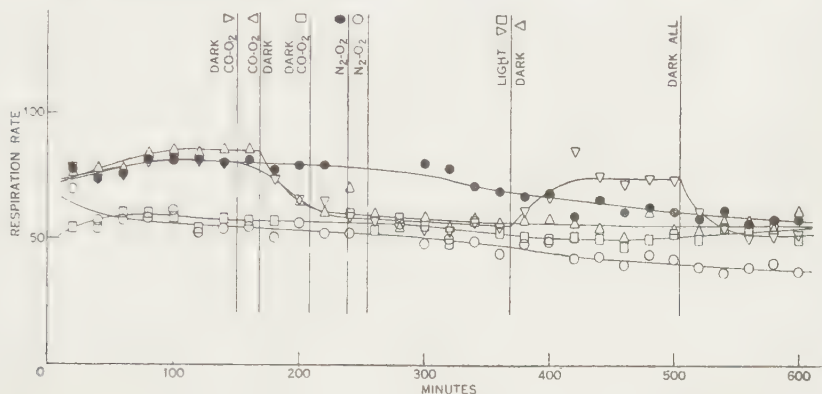


Fig. 3.—Effect of carbon monoxide on the respiration rate of xylem parenchyma in KNO₃ solution and in water.

Respiration rate, cu. mm. O₂/g. f.wt./hr. Discs 1 mm. thick; KNO₃, 0.075M.

Gases 89% CO + 11% O₂ and 91% N₂ + 9% O₂. Time from cutting, 72 hr.

complete at higher concentrations. Cyanide was previously shown to inhibit completely the salt respiration in carrot tissue. Experiments were therefore carried out to determine to what extent the cyanide-sensitive fraction of the

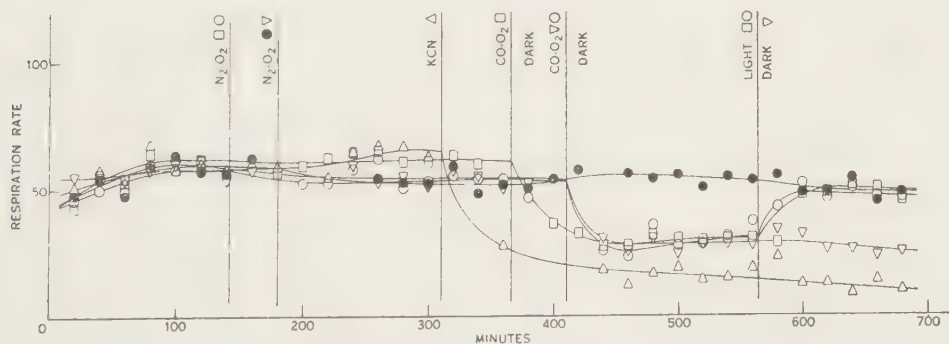


Fig. 4.—Effect of carbon monoxide and KCN on the respiration rate of xylem parenchyma in KCl solution.

Respiration rate, cu. mm. O₂/g. f.wt./hr. Discs 0.75 mm. thick; KCl, 0.1M; KCN, 0.001M.

Gases all contained 5.3% O₂. Time from cutting, 404 hr.

respiration was inhibited by CO. Calculation of the relative affinity of the enzyme towards O₂ and CO helps to identify the enzyme concerned. Warburg (1927) evolved a formula to give an approximate measure of the relative affinity; while the limitations of this formula must be recognized, it has been used

TABLE 2

EFFECTS OF CARBON MONOXIDE IN LIGHT AND DARKNESS ON THE RESPIRATION RATE OF XYLEM PARENCHYMA IN WATER AND IN KCl SOLUTION; THE PARTITION CONSTANT, K, HAS BEEN CALCULATED

Discs 0.75 mm. thick; KCl, 0.1M; KCN, 0.001M; gases all contained 5.3% O₂; time from cutting, Expt. 1, 237 hr., Expt. 2, 404 hr.

		Respiration Rates (cu. mm. O ₂ /g. f. wt./hr.)						K	Percentages						
		N ₂ -O ₂ Initial	N ₂ -O ₂ Final	CO-O ₂ Dark Initial	CO-O ₂ Dark Final	N ₂ -O ₂ at Light	CO-O ₂ Light Initial		KCN Initial	CO-O ₂ Light	CO-O ₂ Dark	CO-O ₂ Light	CO-O ₂ Dark	Mean N ₂ -O ₂	Own N ₂ -O ₂
Experiment 1															
Air	KCl	70.0	—	—	—	—	—	—	—	—	—	—	—	—	—
		73.0	64.5	61.5	28.3	25.8	—	46.1	—	—	—	—	—	—	—
		75.5	—	—	—	—	—	—	—	—	23.5	178.7	110.0°	46.0	—
		74.5	63.2	—	—	—	—	—	—	—	—	—	—	—	—
		69.0	60.3	—	—	—	—	—	—	—	—	—	—	—	—
	Water	57.0	48.5	54.3	34.4	35.0	—	45.9	—	25.7	—	14.0	131.0	121.4°	63.4
		62.5	—	—	—	—	—	—	—	—	—	7.5	—	—	78.3
		61.0	55.1	42.4	33.2	32.7	—	—	—	—	—	10.7	—	—	—
		57.5	51.7	48.4	—	—	—	37.8	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—	—	—	—	—	—
Experiment 2															
Water	66.0	53.7	51.4	22.9	28.5	—	42.5	c. 18	—	149.2	—	44.6	—	—	
	58.5	58.0	55.9	25.8	32.7	—	50.1	—	3.8	153.2	92.0	46.1	—	—	
	62.0	61.7	60.2	25.4	30.8	—	49.2	—	3.1	159.7	90.4	42.0	—	—	
KCl	60.0	54.4	55.2	—	—	—	54.4	—	6.2	—	—	50.9	—	—	
	58.0	58.5	57.4	29.2	—	—	dark	19.4	—	—	—	—	—	—	
	65.0	—	—	—	—	—	—	—	4.4	—	—	—	—	—	

* Caused by the fall in O₂ concentration due to respiration in N₂-O₂.

here because it has been used widely by other authors with whose work these results are to be compared. Warburg showed that substrate saturation of the enzyme (i.e. maximum rate of operation) is necessary to estimate correctly this relative affinity; hence a high salt concentration, 0.1M KCl, was used to ensure maximal salt respiration (Robertson and Wilkins 1948), though we do not know that this necessarily ensures substrate saturation.

The ratio of affinities of the enzyme towards O_2 and CO was expressed by the partition constant, K ,

$$K = \frac{K_{O_2}}{K_{CO}} = \frac{n}{1-n} \cdot \frac{P_{CO}}{P_{O_2}},$$

where 1 = the total cyanide-sensitive respiration in the control N_2 - O_2 atmosphere, and n = the fraction not inhibited by CO; P_{CO} and P_{O_2} are the partial pressures of the gases in the mixture. Since an appreciable fraction of the respiration of carrot cells is cyanide-stable, K was calculated from the cyanide-sensitive respiration alone. Some data, with the respective values for K , are given in Table 2. A typical experiment yielding such data is illustrated in Figure 4. The cytochrome oxidase mediating the respiration of yeast has a partition constant of from 4 to 13, depending on the extent of inhibition of the respiration (Warburg 1927). The "constant" decreases as the percentage inhibition increases (Winzler 1943). This is because Warburg's equation does not allow for the relative capacities and rates of the individual reactions comprising the chain of terminal oxidation processes; thus K is not the true O_2 -CO partition constant but varies with the extent of inhibition. It is seen that the values of K based on the cyanide-sensitive respiration in carrot tissue lie within the same approximate limits, 2.5-6.2 for tissue in salt solution and 7.5-14 for tissue in water. According to Oppenheimer and Stern (1939), under conditions in which cytochrome oxidase gives values of 7-30 for the ratio CO: O_2 at 50 per cent. inhibition, polyphenol oxidase gives a value of 1.5. Levy and Schade (1948) found that an 80 per cent. CO-20 per cent. O_2 mixture completely inhibited the action of potato tyrosinase *in vitro*, while inhibiting by less than 20 per cent. the cytochrome oxidase-carried respiration of potato tissue slices. The above discussion indicates that the cyanide-sensitive respiration in carrot tissue could be mediated by cytochrome oxidase.

Values of K calculated from CO-inhibition of the total respiration, however, were more variable and always high; from inhibition of the total respiration in water in particular, values often approached 100 or more; from inhibition of the total respiration in salt solution, values ranged from 19 to 29 with a mean value at 24.

(f) *Light Reversibility of the CO Inhibition of Respiration.*

It is shown clearly in Tables 1 and 2 and Figures 2, 3, and 4, that the respiratory inhibition by CO in darkness is reversed by strong light. In the experiments where the O_2 concentration was the same in CO- O_2 and N_2 - O_2 mixtures, the reversal caused by the full mercury light was almost complete, the respiration rates returning to within 80 to 100 per cent. of the control rates.

Blue light is effective in reversing CO inhibitions, but red light of wavelengths greater than $590\text{ m}\mu$ is not effective, as shown in Table 3. While the red light was of lower intensity than the blue, a partial reversal of inhibition relative to the blue effect would have been expected if the spectrum of the inhibited enzyme had displayed strong absorption bands in the red. The CO-enzyme complex evidently absorbs the blue light (which consists principally of $436\text{ m}\mu$) strongly, but does not absorb the red at all. This is in agreement with the reported spectrum of the cytochrome oxidase-CO complex (Melnick 1942).

TABLE 3
EFFECTS OF CARBON MONOXIDE IN DARKNESS AND IN RED AND BLUE LIGHT ON THE RESPIRATION RATE OF XYLEM PARENCHYMA

Discs 0.75 mm. thick; KNO_3 , 0.075M

Expt. 1: gas, 93% CO + 7% O_2 ; blue light; time from cutting, 69 hr.

Expt. 2: gas, 95% CO + 5% O_2 ; red and blue light; time from cutting, 165 hr.

Expt. 3: gas, 95% CO + 5% O_2 ; red and blue light; time from cutting, 236 hr.

Respiration Rates (cu. mm. O_2 /g. f. wt./hr.)				Percentage	
Air	CO- O_2 Dark Initial	CO- O_2 Dark Final	CO- O_2 Light Initial	CO- O_2 Light Own CO- O_2 Dark	
Experiment 1					
KNO_3 {	54.5	29.9	29.4	Dark	
	42.0	36.9	35.1	46.2	131.6
	47.2	31.2	31.2	42.2	135.3
H_2O	39.5	39.9	30.6	39.9	130.4
Experiment 2					
KNO_3 {	94.2	66.8	67.8	67.8	100
	93.2	70.6	72.2	72.2	100
	101.5	86.6	78.1	79.5	101.8
	90.8	91.5	85.2	106.9	125.5
					} Red
					} Blue
Experiment 3					
KNO_3 {	119.0	101.7	100.3	93.8	93.5
	108.5	90.7	81.1	73.0	90.0
	110.0	87.0	73.0	—	—
	102.0	105.2	90.6	105.1	116.0
	110.5	94.6	90.1	99.0	109.9
					} Red
					} Blue

(g) CO Inhibition of Accumulation

The accumulation of salt ions by the cells was shown previously (Robertson and Turner 1945) to be inhibited completely by KCN. The effect of CO

upon accumulation has not previously been investigated°. Experiments given in Table 4 and also illustrated by Figure 5 showed that accumulation was markedly inhibited by CO and that the inhibition was reversed by light.

TABLE 4

EFFECT OF CARBON MONOXIDE ON THE RATE OF ACCUMULATION OF KCl BY XYLEM PARENCHYMA; THE PARTITION CONSTANT, K , HAS BEEN CALCULATED

Discs 0.75 mm. thick; KCl, 0.1M; gases all contained 8% O₂; time from cutting, Expt. 1, 163 hr., Expt. 2, 210 hr.

Accumulation Rates (g. mol. KCl/g. f. wt./hr. × 10 ⁵)					Percentages			
Air	CO-O ₂ Dark	CO-O ₂ Light	N ₂ O ₂	K	CO-O ₂ Dark Mean N ₂ -O ₂	CO-O ₂ Light Mean N ₂ -O ₂	CO-O ₂ Light CO-O ₂ Dark	
Experiment 1								
0.600	0.0974	—	—	4.4	27.8	—	—	
0.503	—	—	0.366	—	—	—	—	
0.570	—	—	0.333	—	—	—	—	
0.619	0.0712	0.262	—	2.9	20.3	74.9	368	
				3.7				
Experiment 2								
0.574	—	—	0.359	—	—	—	—	
0.574	0.116	0.270	—	5.6	32.8	76.1	232	
0.539	0.106	—	—	4.9	30.0	—	—	
0.561	—	—	0.351	—	—	—	—	
				5.3				

Partition constants for the ratio of affinities to O₂ and CO of the CO-sensitive enzyme upon which accumulation is dependent were calculated by Warburg's formula, as above. The partition constants (2.9-5.6) are similar to those obtained for the cytochrome oxidase mediating the salt respiration. Hence, as had been previously assumed, both the ion accumulation mechanism and the salt respiration in carrot root tissue are dependent upon the cytochrome system.

IV. DISCUSSION

These results provide the link between the work of Marsh and Goddard (1939) and that of Robertson and Turner (1945). The assumption made earlier that the cyanide-sensitive salt respiration in carrot root cells is mediated by the cytochrome system is evidently correct. All the above evidence indicates that the cytochrome-cytochrome oxidase system and no other oxidase

° A personal communication from Professor H. Lundegardh received while this paper was being prepared indicates that this has recently been investigated in his laboratory.

system at present known is involved. That cytochrome oxidase is present in carrot tissue has been shown recently by Rowan* (personal communication), thus confirming earlier unpublished reports (see Robertson and Wilkins 1948). Rowan has demonstrated the presence of a cytochrome oxidase in the suspended sediment obtained from strained, centrifuged carrot root brei. It was active in oxidizing *p*-phenylenediamine only in the presence of added cytochrome *c*; the oxidation was photoreversibly inhibited by CO and the partition constant was approximately 4.

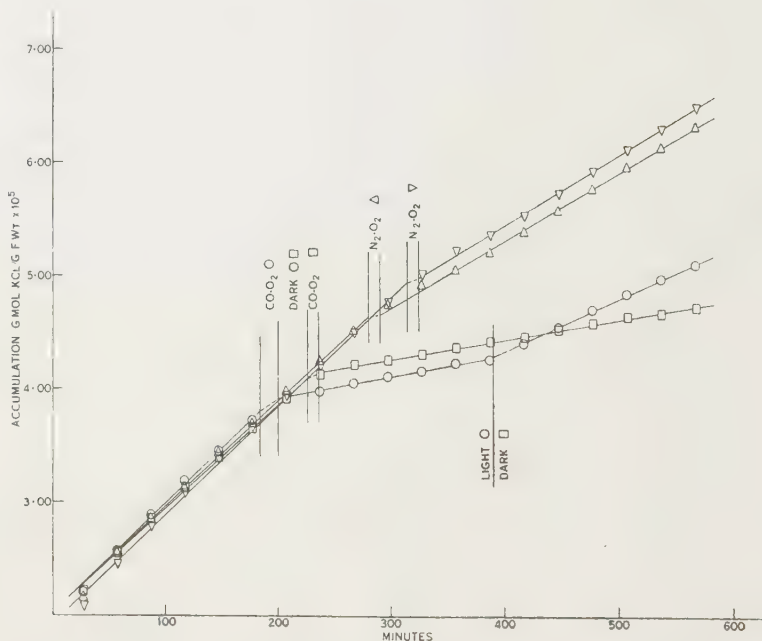


Fig. 5.—Effect of carbon monoxide on the rate of accumulation of KCl by xylem parenchyma. Accumulation, g. mol. KCl/g. f.wt. $\times 10^5$. Discs 0.75 mm. thick; KCl, 0.1M. Gases all contained 8% O_2 . Time from cutting, 210 hr.

Data from CO inhibition of the cyanide-sensitive fraction of the respiration of discs in water yield values of *K* greater than those for the respiration in salt solutions. This is due to the operation of only a fraction of the total available cytochrome oxidase, and is presumably equivalent to lack of substrate saturation.

The high values of the partition constant *K* when calculated from CO-inhibition of the total respiration are due to the inclusion of CO-stable ground respiration in the values for the residual fraction *n*, since, on the tissue used here, the ground respiration is usually a major part of the total in the presence of CO. Marsh and Goddard (1939) have previously obtained values of the partition constant for CO inhibition of the total respiration of carrot slices in phosphate

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buffer. They are higher than those found here for the cyanide-sensitive fraction alone, due to the inclusion of the ground respiration in values of n , but they are lower than those obtained here for the total respiration in the presence of salt. It is evident that, in their carrots (var. Nantes Coreless), the ground respiration is a distinctly smaller fraction of the total than in those used here. Only two figures are given for the ground or cyanide-stable respiration in the "Type A" carrots used for their carbon monoxide experiments; these were obtained with tissue in $8 \times 10^{-4}M$ KCN, a concentration just sufficient to produce maximal inhibition in the tissue used here. In Table 5, their data and data from several of the authors' experiments with tissue in KCl solution are compared. It is clear that values of K obtained from the cyanide-sensitive fraction alone are quite similar for the two tissues. Thus the cytochrome oxidases operating in the two varieties of carrot can be considered similar in nature. The presence of a large cytochrome oxidase fraction in the respiration of their freshly cut tissue is evidently due in part to its immersion in phosphate buffer, inducing a small salt respiration, and also in part to the wound effect following cutting. In relation to wound effects, it is significant that their discs were cut by hand to a thickness of 0.5 mm., having thus a much larger surface to volume ratio than those used here.

TABLE 5
COMPARISON OF RESPIRATION AND PARTITION CONSTANTS

Carrot Tissue var.	$\mu\text{l.}/\text{O}_2/\text{g. f.wt.}/\text{hr.}$			Partition Constants (K) Calculated from:	
	Mean Total Respiration in 95% $\text{N}_2 + 5\% \text{O}_2$	Mean Total Respiration in 95% $\text{CO} + 5\% \text{O}_2$	Mean Respi- ration in KCN Solution	Total Respiration	Cyanide- Sensitive Respiration
Nantes Core- less (Marsh and Goddard 1939)	62.0	22.3	12.8 ($8 \times 10^{-4}M$ KCN)	10.7	4.6
Danver's Half-Long	<i>c.</i> 50	<i>c.</i> 30	<i>c.</i> 23 ($1 \times 10^{-3}M$ KCN)	28.5	6.6

The stability of the ground respiration towards carbon monoxide and cyanide and the high partition constants obtained from data on CO-inhibition of total respiration indicate that the ground respiration is not mediated by the cytochrome system. This means either that the ground respiration is mediated by an oxidase system other than cytochrome oxidase or that, as Ball (1942) suggests, the cytochrome oxidase-cyanide and the cytochrome oxidase-carbon monoxide complexes might continue, with their reduced redox potentials, to effect the oxidation of flavoprotein directly without the mediation of the cytochrome pigments. This might account for an apparent ground respiration in the presence of cyanide, but the carbon monoxide-oxidase complex could not

operate in this manner, which suggests that the ground respiration is mediated by a system other than cytochrome oxidase. In any event, both cyanide and carbon monoxide inhibit the normal operation of the complete cytochrome respiratory system as such—i.e. the stepwise passage of electrons from reduced flavoproteins along the chain of the cytochromes finally to the oxidase and oxygen—which would be sufficient to account for the inhibition of accumulation, according to Lundegårdh's hypothesis.

The accumulatory mechanism is evidently dependent on the energy of the salt respiration mediated by the cytochrome oxidase system, which does not seem to be involved in the ground respiration. Under the conditions of the present experiments, the accumulation of KCl is inhibited by CO in the same way, quantitatively, as the salt respiration which is directly linked to the cytochrome system. The results reported here are thus consistent with Lundegårdh's hypothesis of ion accumulation (1945).

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